"PHYTOCHEMICAL EVALUATION AND ANTICANCER ACTIVITY ON ROOT EXTRACTS OF *JATROPHA GOSSYPIIFOLIA* LINN.,"

A dissertation submitted to

THE TAMIL NADU Dr. M. G. R MEDICAL UNIVERSITY

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In partial fulfilment of the requirements for the award of degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL CHEMISTRY

Submitted by 261415715

Under the guidance of

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CERTIFICATE

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Praise the bridge that carried you over.

-George Colman

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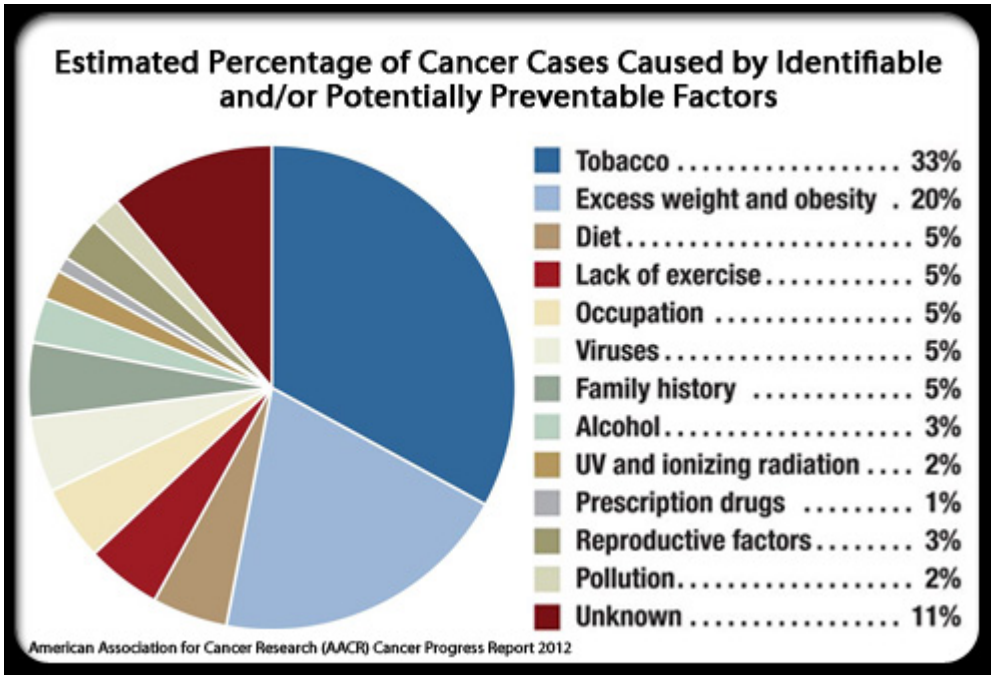
CONTENTS

1. INTRODUCTION

Cancer, in medicine, common term for neoplasms, or tumors, that are malignant. Like benign tumors, malignant tumors do not respond to body mechanisms that limit cell growth. Unlike benign tumors, malignant tumors consist of differentiated, or unspecialized, cells that show an atypical cell structure and do not function like the normal cells from the organ from which they derive. Cancer is the major disorder, which increases morbidity and mortality in both male and females[1,2]

Causes of Cancer

Fig 1. Percentage of cancer causes by different factors



Causes of Cancer

Certain genes control the life cycle – the growth, function, division, and death -- of a cell. When these genes are damaged, the balance between normal cell growth and death is lost. Cancer occurs due to DNA damage and out-of-control cell growth. The following is a partial list of factors known to damage DNA and increase the risk of cancer:

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- Genetic mutations (for example, BRCA1 and BRCA2)
- Environmental exposure to UV radiation, air pollution
- Bacterial (H. pylori) and viral infections (Epstein-Barr, HPV, hepatitis B and C)
- Lifestyle choices (poor diet, inactivity, obesity, heavy alcohol use, smoking cigarettes and tobacco use, exposure to chemicals and toxins)
- Treatment with chemotherapy, radiation, or immunosuppressive drugs. [3,4]

Gene Mutation

Cancer is caused by changes (mutations) to the DNA within cells. The DNA inside a cell is packaged into a large number of individual genes, each of which contains a set of instructions telling the cell what functions to perform, as well as how to grow and divide. Errors in the instructions can cause the cell to stop its normal function and may allow a cell to become cancerous.

Effects of gene mutations

A gene mutation can instruct a healthy cell to allow rapid growth. A gene mutation can tell a cell to grow and divide more rapidly. This creates many new cells that all have that same mutation.

1. Failure to stop uncontrolled cell growth.
2. Normal cellsknow when to stop growing so that you have just the right number of each type of cell. Cancer cells lose the controls (tumor suppressor genes) that tell them when to stop growing. A mutation in a tumor suppressor gene allows cancer cells to continue growing and accumulating.
3. Make mistakes when repairing DNA errors. DNA repair genes look for errors in a cell's DNA and make corrections. A mutation in a DNA repair gene may mean that other errors aren't corrected, leading cells to become cancerous.

These mutations are the most common ones found in cancer. But many other gene mutations can contribute to causing cancer.

Causes gene mutations

Gene mutations can occur for several reasons, for instance:

Gene mutations you're born with. You may be born with a genetic mutation that you inherited from your parents. This type of mutation accounts for a small percentage of cancers.

Gene mutations that occur after birth. Most gene mutations occur after you're born and aren't inherited. A number of forces can cause gene mutations, such as smoking, radiation, viruses, cancer causing chemicals (carcinogens), obesity, hormones, chronic inflammation and a lack of exercise.

Gene mutations occur frequently during normal cell growth. However, cells contain a mechanism that recognizes when a mistake occurs and repairs the mistake. Occasionally, a mistake is missed. This could cause a cell to become cancerous.[5,6]

Symptoms of Cancer

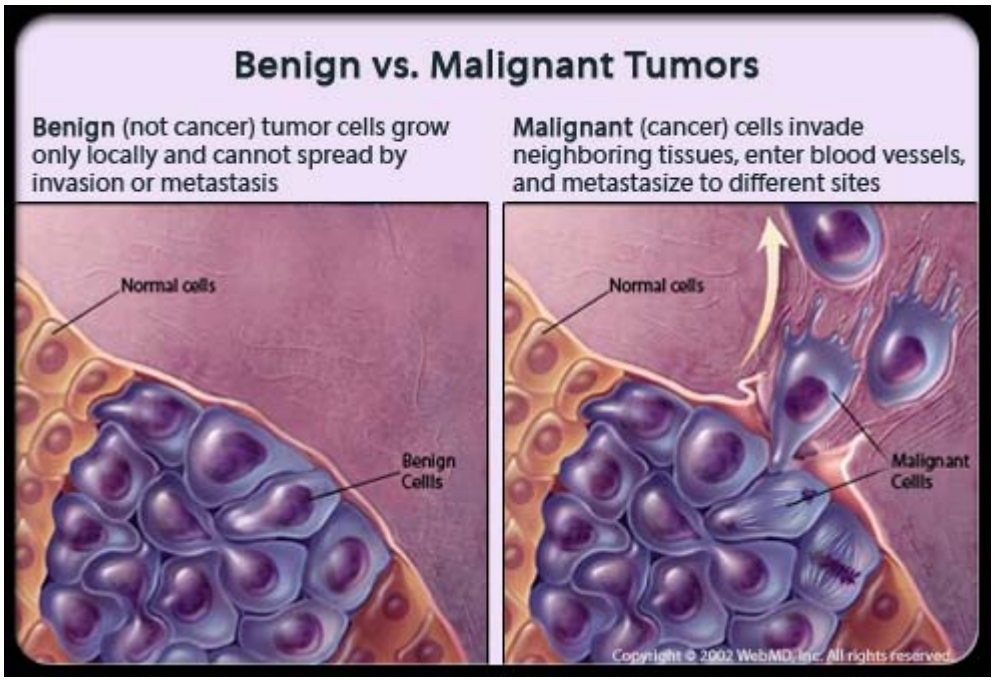
Signs and symptoms caused by cancer will vary depending on what part of the body is affected.[4]

Some general signs and symptoms associated with, but not specific to, cancer, include:

- Fatigue
- Lump or area of thickening that can be felt under the skin
- Weight changes, including unintended loss or gain
- Skin changes, such as yellowing, darkening or redness of the skin, sores that won't heal, or changes to existing moles
- Changes in bowel or bladder habits
- Persistent cough or trouble breathing
- Difficulty swallowing
- Hoarseness
- Persistent indigestion or discomfort after eating
- Persistent, unexplained muscle or joint pain
- Persistent, unexplained fevers or night sweats
- Unexplained bleeding or bruising

Types of Cancer

Fig 2. Benign Vs. Malignant Tumors



Tumors: Benign vs. Malignant

A tumor is an abnormal mass of cells. Tumors can either be benign (non-cancerous) or malignant (cancerous). Benign tumors grow locally and do not spread. Malignant tumors have the ability to spread and invade other tissues. This process, which is a key feature of cancer, is known as metastasis.

Types of Cancer

There are over 200 types of cancer. However, the NCI lists several general categories . It is not all inclusive and the cancers listed in quotes are the general names of some cancers:

Carcinoma: Cancer that begins in the skin or in tissues that line or cover internal organs -- "skin, lung, colon, pancreatic, ovarian cancers," epithelial, squamous and basal cell carcinomas, melanomas, papillomas, and adenomas.

Sarcoma: Cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue -- "bone, soft tissue cancers," osteosarcoma, synovial sarcoma, liposarcoma, angiosarcoma, rhabdosarcoma, and fibrosarcoma.

Leukemia: Cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood -- "leukemia,"

lymphoblastic leukemias (ALL and CLL), myelogenousleukemias (AML and CML), T-cell leukemia, and hairy-cell leukemia.

Lymphoma and myeloma: Cancers that begin in the cells of the immune system -- "lymphoma," T-cell lymphomas, B-cell lymphomas, Hodgkin lymphomas, non-Hodgkin lymphoma, and lymphoproliferative lymphomas

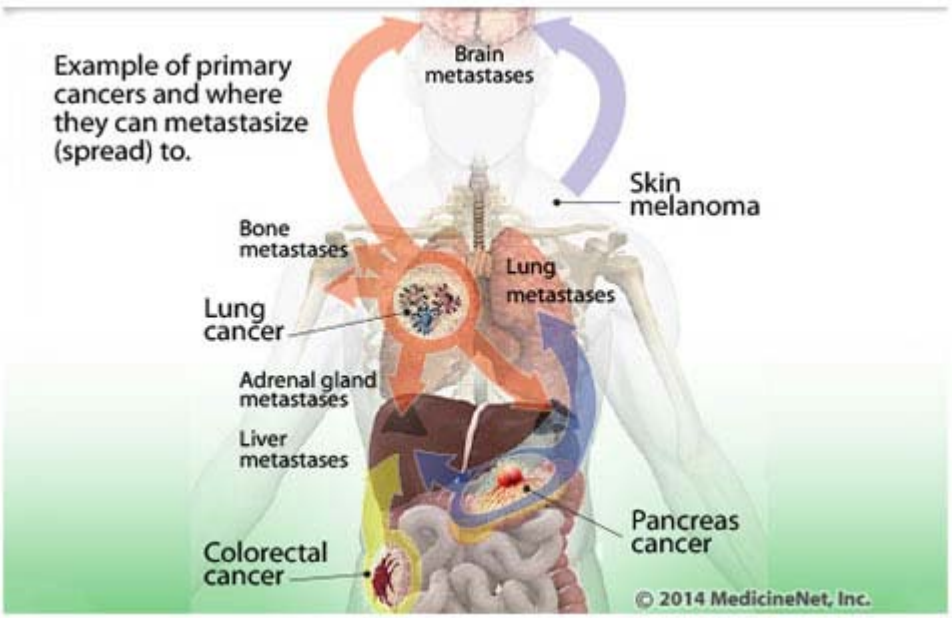
Central nervous system cancers: Cancers that begin in the tissues of the brain and spinal cord -- "brain and spinal cord tumors," gliomas, meningiomas, pituitary adenomas, vestibular schwannomas, primary CNS lymphomas, and primitive neuroectodermal tumors.[7]

Metastasis

Metastasis is the process whereby cancer cells break free from a tumor and travel to and invade other tissues in the body. Cancer cells metastasize to other sites via the lymphatic system and the bloodstream. Cancer cells from the original -- or primary -- tumor can travel to other sites such as the lungs, bones, liver, brain, and other areas. These metastatic tumors are "secondary cancers" because they arise from the primary tumor.

Metastatic cancer retains the name of the primary cancer. For example, bladder cancer that metastasizes to the liver is not liver cancer. It is called metastatic bladder cancer. Metastasis is significant because it helps determine the staging and treatment of cancer. Some types of metastatic cancer are curable, but many are not.

Fig 3. Example of primary cancer metastasize



Not included in the above types listed are metastatic cancers; this is because metastatic cancer cells usually arise from a cell type listed above and the major difference from the above types is that these cells are now present in a tissue from which the cancer cells did not originally develop. Consequently, if the terms "metastatic cancer" is used, for accuracy, the tissue from which the cancer cells arose should be included. For example, a patient may say they have or are diagnosed with "metastatic cancer" but the more accurate statement is "metastatic (breast, lung, colon, or other type) cancer with spread to the organ in which it has been found.[8,9]

Cancer - Indian scenario

Every year about 8, 50,000 new cancer cases being diagnosed, India resulting about 5, 80,000 cancers related death every year. India had the highest number of the oral and throat cancer cases in the world. Every third oral cancer patient in the world is from India. In males Oral, Lungs and Stomach cancers was the three most common causes of cancer incidence and death whereas In females Cervical, Breast and Oral cancers were the three main causes of cancer related illnesses and death.

Overall cervical cancer was the number one cause of cancer death in India. That was really unfortunate as cervical cancer can be easily prevented and also relatively easy to diagnose and treat at an early stage. Compared to developed countries overall there were less cancer cases in India but that could be due to under diagnosis and under reporting. At the same time regional, ethnic, dietary and socio-economic factors might also results in difference in the cancer susceptibilities and the incidence. Also cancer was mainly a disease of old ages. Worldwide median age at diagnosis was about 60 years. Average life span was about 58 yrs in India comparedto 75 yrs in the developed world.

Cancer- global scenario

Among all the cancer, Lung cancer is the most common worldwide and accounts for major deathannually. The three leading cancer killers were different than the three most common forms, (i) Lung cancer responsible for 17.8 per cent of all cancer deaths. (ii) Stomach 10.4 per cent and (iii) Liver 8.8 percent. Industrial nations with the highest overall cancer rates include: U.S.A, Italy, Australia, Germany, The Netherlands, Canada and France. Developing countries with the lowest cancer were in Northern Africa. Cancer rates could further increase by 50% to 15 million new cases in the year 2020.According to the World Cancer Report, the most comprehensive global examination of the disease to date. However, the report also provides clear evidence that healthy lifestyles, and publichealth action by governments and

health practitioners could stem this trend, thus prevent as manyas one third of cancers worldwide.[10]

Table 1.Type of cancer and No. of patients affected/year

S.No	Type of Cancer	No. of Patients affected /year
1.	Lung	1.2 million
2.	Breast	Over 1 million
3.	Colorectal	9,40,000
4.	Stomach	8,70,000
5.	Liver	5,60,000
6.	Cervical	4,70,000
7.	Esophageal	4,10,000
8.	Head and Neck	3,90,000
9.	Urinary Bladder	3,30,000
10.	Malignant Non-Hodgkin lymphomas	2,90,000
11.	Leukemia	2,50,000
12.	Prostate and Testicular	2,50,000
13.	Pancreatic	2,16,000
14.	Ovarian	1,90,000
15.	Kidney	1,90,000
16.	Endometerial	1,88,000
17.	Nervous system	1,75,000
18.	Melanoma	1,33,000
19.	Thyroid	1,23,000
20.	Pharynx	65,000

Treatment

Developments in the treatment of cancer have led to greatly improved survival and quality of life for cancer patients in the past three decades. Traditionally, cancer has been treated by surgery, chemotherapy, and radiation therapy. In recent years immunotherapy has been added to that list. New drugs and techniques are constantly being researched and developed, such as antiangiogenic agents (e.g., angiostatin and endostatin), genetically engineered monoclonal antibodies, retinoid agents, and therapeutic vaccines (agents that stimulate the immune system to attack cancerous cells).

Radiation therapyeither from an external beam or from implanted radioactive pellets—is the primary treatment. The usual forms are X rays and gamma rays. Use of radioactive elements specific for particular target organs, such as radioactive iodine specific for the thyroid gland, is effective in treating malignancies of those organs.

Cytotoxic chemotherapy is used as a primary treatment for some cancers, such as lymphomas and leukemias or as an addition to surgery or radiation therapy. Cytotoxic drugs (drugs that are toxic to cells) are aimed at rapidly proliferating cells and interfere with nucleic acid and protein synthesis in the cancer cell, but they are often toxic to normal rapidly proliferating cells, such as bone marrow and hair cells.

Hormonal chemotherapy Is based upon the fact that the growth of some malignant tumors (specifically those of the reproductive organs) is influenced by reproductive hormones. Tamoxifen is a naturally occurring estrogen inhibitor used to prevent breast cancer recurrences. Flutamide is sometimes used in prostate cancer to inhibit androgen uptake. Sex-hormone related drugs such as DES and tamoxifen, which may be carcinogenic under some conditions, have proven to be protective under others.

Immunotherapy (sometimes called biological therapy) uses substances that help the body mobilize its immune defenses. Some attack the tumor itself, while others bolster the body's ability to withstand conventional chemotherapy treatment. Other new or experimental therapies include drugs that inhibit angiogenesis and photodynamic therapy, in which a patient is given a drug to make the tumor light-sensitive, after which the tumor is exposed to bright laser light.[11]

Phytochemical importance of medicinal plants as potential sources of anticancer agents

The potential medicinalproperties of plant species have contributed significantly in the development of various herbal therapies for a number of diseases acrossthe globe. The benefits of herbal medicine over allopathic medicine have helped medicinal plants to regain their importance in the fieldof health and medicine. Cancer is one of the major health problems that have widely affected the world's population. There is a greatneed to combat this disease with better and more effective medication as compared to existing therapies. A vast number of medicinalplants are known to have biochemical constituents with anticancer properties. The chemical metabolites of natural origin that possessanticancer properties can serveas potential lead compounds in drug designing. This association of medicinal plants and cancer needsfurther research and experimentation in order to develop and design anticancer drugs.

Thevast array of therapeutic effects associated with medicinal plants includes anti-inflammatory, antiviral, antitumor,antimalarial, and analgesic. Cancer is one of the majorobstacles to human health around the world. Among allepidemic diseases, cancer holds the first place as a deathcausingdisease. The main reason behind the growingnumber of cancer cases is the changing lifestyle of thepopulation across the globe. Keeping in view the statisticaldata, the most prevalent cancer among females is breastcancer, accounting for about 23% of total cancer cases; inmales, the most prevalent is lung cancer, which accountsfor 17% of total cancer cases[12]. Poorsurvival rate of cancer patients in developing countriesis attributed to the lack of timely diagnosis and limitedtreatment facilities. There is a great need to address thisepidemic disease with more effective therapeutic andpreventive strategies, which could be possible with the useof natural compounds.Recently the scientific world has experienced anupsurge of interest in the therapeutic potential of medicinalplants as a source of promising anticancer agents. However,the application of plant-based compounds for the treatmentof cancer can be traced back to 1950s. Some of the very firstanticancer agents derived from plants are vinca alkaloids,vinblastine, vincristine, and cytotoxic podophyllotoxins.Statistical data suggest that 16 plant-derived anticancer drugshave been subjected to clinical trials so far [13]. Landmarks of these clinical trials are flavopiridol,isolated from the Indian tree *Dysoxylumbinectariferum*,andmeisoindigo, isolated from the Chinese plant *Indigoferatinctoria*, which have been documented to have less toxicitythan

conventional chemotherapeutic anticancer drugs.[14] These discoveries have propelledthe scientific interest of various research groups in thediscovery of new anticancer agents from all-natural productsources, inclusive of plant secondary metabolites. Theemerging importance of natural anticancer agents demandsmore research and experimentation in order to developsuccessful natural therapeutic options for this disease.

Medicinal plants as the best choice for cancer treatment

The chemical components of medicinal plants mainly possess antioxidant properties that contribute to their anticancer potential. Flavones, isoflavones, flavonoids, anthocyanins, coumarins, lignans, catechins, andisocatechins are the major classes of bioactive constituents responsible for the antioxidant action.[15] The great potential of plant-based compounds for the treatment and prevention of cancer is attributed totheir safety, low cost, and oral bioavailability. The already available expensive conventional therapies for cancer like chemotherapy and radiotherapy have a number of side effects such as myelosuppression and neurological, cardiac, pulmonary, and renal toxicity, which pose serious harm to the quality of life.[16]

Flavonoids

Flavonoids may act at the different development stages of malignant tumors by protecting DNA against oxidative damage, inactivating carcinogens, inhibiting the expression of the mutagenic genes and enzymes responsible for activating procarcinogenic substances, and activating the systems responsible for xenobiotic detoxification.[17]

Although most flavonoids appear to be nontoxic to humans and animals, they have been demonstrated to inhibit proliferation in many kinds of cancerous cell lines. For instance, it has been reported that flavonoids (quercetin and taxifolin) have anti-proliferative effects on squamous cell carcinoma HTB43.[18]Quercetin at 10 μM shows an anti-proliferative activity against meningioma cells [19] and against colon cancer cells (Caco-2 and HT-29), with a dose-ependent effect.[20]Diosmin, another important Citrus flavonoid, which is on the market as a venotonic, has shown anti-proliferative activity in Caco-2 and HT-29 colon cancer cell lines (IC_{50} 203 μM), although with less efficacy than quercetin.[21]

Therefore, there is a need to develop treatment options that include more potent and less toxic anticancer drugs as compared to existing drugs. The market statistics mark the availability of approximately 60% plant-based anticancer drugs.[22] Medicinal plants constitute a common

alternative to cancer treatment in many countries of the world.[23,24] Cytotoxic screening of a number of plants has been done to correlate their anticancer activity and further expand their scope for drug development.[25]Owing to potential benefits of plantbased drugs for cancer treatment, their use is increasingly growing from 10% to 40% across the globe; specifically, on the Asian continent, it has reached 50%. Anticancer benefits associated with natural plant derivatives demand extensive scientific screening and clinical experimentations for thedevelopment of improved drugs.[26,27]

2.REVIEW OF LITERATURE

Pharmacological Review

1. Sergio Granados et al., (2015) reported a bio guided assay, a crude extract stimulated glucose uptake in C2C12 myotubes up to 30%, thereby reducing insulin resistance induced by fatty acids compared to the basal control. A chromatographic fraction applied intraperitoneally (IP) in mice reduced glucose by 42% in a mouse model of T2DM, after administration of 10 doses during 20 days. A flavanone was purified from this active fraction and its structure was assigned by ^{1}H- and ^{13}C-NMR (1D and 2D) and MS. This compound retains the previously reported activity, stimulating *in vitro* the glucose uptake in a concentration-dependent manner.[28]

2. Murugalakshmi et al., (2014) investigated purgative activities of ethyl alcohol and acetone extracts using albino rats of both the sexes. The phytochemicalscreening of ethanoic extracts of *Jatropha gossypiifolia* leaves showsthe presence secondary metabolites such as Saponin, Tannins, Phenols, Steroids, Glycosides,Flavonoids etc., The ethanoic and acetone extract of *Jatropha gossypiifolia* have significantantipyretic activity and purgative activity.[29]

3. Juliana Félix-Silva et al., (2014) reported a review article, onup-to-date overview of the traditional uses, as well as the phytochemistry, pharmacology, and toxicity data of *J. gossypiifolia* species, in view of discussing its medicinal value and potential application in complementary and alternative medicine. Pharmacological studies have demonstrated significant action of different extracts and/or isolated compounds as antimicrobial, anti-inflammatory, antidiarrheal, antihypertensive, and anticancer agents, among others, supporting some of its popular uses.Toxicological studies associated with phytochemical analysis are important to understand the eventual toxic effects that could reduce its medicinal value.[30]

4. VidhyaKumari and Shikha Roy (2014) investigated the antibacterial activity of crude chloroform, methanolic and aqueous extracts of *Jatropha gossypiifolia* L stem and leaves was investigated using disc agar diffusion against clinical isolates of bacteria consisting of Bacillus subtilis, Escheria coli and Pseudomonas putida *Jatrophagossypiifolia* L well known plant of family Euphorbiaceae is used as a therapeutic agent. Crude powder of stem

and leaves screened for antibacterial activity by zone of inhibition. The extracts exhibited broad spectrum activities against microorganisms.[31]

5. YerramsettyNagaharika et al., (2013) evaluated the anti-inflammatory activity of aqueous and alcoholic extract of *J. gossypiifolia* leaves by *in vitro* HRBC membrane stabilization method. The *in vitro* method showed significant anti-inflammatory property of different extracts tested. The aqueous extract at a concentration of 200g/mL showed significant activity when compared with the standard drug diclofenac sodium.[32]

6. ApurbaSarkerApu et al., (2013)*Solanumsisymbriifolium* (Solanaceae) and *Jatrophagossypiifolia* (Euphorbiaceae) were evaluated for cytotoxic activity. Methanol crude extract and fractions of the extract of leaves of *Jatropha gossypiifolia* exhibited significant toxicity against the shrimps (LC_{50} values 17.19 – 98.19 μg/ml), while that of potassium permanganate, a positive control, was 11.27 μg/ml. Among the fractions of the crude extracts of both plants, non-polar fractions such as *n*-hexane, chloroform, dichloromethane were the most cytotoxic.[33]

7. Olabinri BM Oladele AP et al., (2013) reported the influence of season , solvent type and concentration on the phenolic and flavonoid contents including *in vitro* antioxidant and nitric oxide radical scavenging activities of stem bark and leaf extracts of fignut (*Jatrophagossypiifolia*) were investigated. Season, extraction solvent type and concentration were critical determinants of the total phenolic and flavonoid contents including *in vitro* antioxidant and nitric oxide radical scavenging activities of the plant parts.[34]

8. Apu et al., (2013) evaluated analgesic activity, measured by acetic acid induced writhing inhibition test. The neuropharmacological activities were evaluated by hole cross, hole-board, and elevated plus-maze (EPM) tests and the anti-diarrheal activity was assessed by castor oil induced diarrhea inhibition method.[35]

9. Harneet Singh and Surendra Kr. Sharma (2013)evaluated the hypoglycemic effect of successive extracts of the roots of the plant in comparison to metformin. Petroleum ether (60-80° C), chloroform, methanol and aqueous extracts (250 and 500 mg/kg body weight)

were orally administered to alloxan induced diabetic mice for 7 days. Change in body weight and plasma glucose levels were estimated after the treatment.[36]

10. Vishnu Sharma et al., (2013) examined the antibacterial activity of aqueous and ethanol extract of *J. gossypiifolia* using the disc diffusion method. Extracts were tested against both Gram positive (Bacillus subtilis MTCC 441 and Staphylococcus aureus MTCC 3381) and Gram negative (Escherichia coli MTCC 1562 and Pseudomonas fragi MTCC 2458) bacteria.[37]

11. Pratibha Singh et al .,(2012) elucidated the possible toxic effects and mortality caused by crude latex and compound apigenin isolated from leaves of *J. gossypiifolia* on freshwater snakehead fish, Channapunctatus and Swiss albino mice Mus musculus.[38]

12. Khumrungsee Net al (2011) evaluated the insecticidal effects of Thai botanical, senescent leaf *Jatropha gossypiifolia* extracts on second instar *Spodopteraexigua* larvae by the dipping method and topical sprayer method. The leaf crude extract was extracted using Soxhlet apparatus with ethyl acetate as solvent. The result shows 60 percent mortality of this parasite species at dose up to 40,000 ppm. Thus, *Jatropha gossypiifolia* leaf crude extracts can be as alternative IPM control tool for *Spodopteraexigua* which friendly to benefit insect such as *Meteorus pulchricornis*.[39]

13. Datta A Dhale et al; (2010) reported antimicrobial effect of *Jatropha gossypiifolia* Linn. (Euphorbiaceae) leaf extract was evaluated on microbial strains like gram-positive species staphylococcus spp., and Bacillus spp. and gram-negative species Echerichia Spp. and Pseudomonas spp. The solvent used for extraction of plant were Petroleum ether, Alcohol, Chloroform. The alcoholic extract of leaves of *Jatropha gossypiifolia* shows maximum antimicrobial activity. The *in vitro* antimicrobial valuation was carried out by agar disc antimicrobial and preliminary phytochemical studies of *Jatropha gossypiifolia*were also performed.[40]

14. B.B. Panda et al., (2009) investigated hepatoprotective activity in carbon tetrachloride induced liver damage in Wister albino rats in the aerial part of *Jatropha gossypiifolia*. The extracts at dose of 200mg/kg were administered orally once daily. The substantially

elevated serum enzymatic levels of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), serum alkaline phosphatase (ALP), total bilirubin, SOD and catalase were restored towards normalization significantly by the extracts.[41]

15. Taofeqoduola et al., (2005) evaluated the anticoagulant for biochemical and hematological analyses. The anticoagulant effect of the extract was found to be highest at a concentration of 0.1 ml per ml of blood. Blood tubes with dried leaf extract at the stated concentration were used for obtaining plasma. Mean plasma glucose values obtained from fluoride oxalate plasma were compared with values obtained from dried leaf extract plasma. The values obtained for biochemical parameters with the exception of bicarbonate from the leaf extract plasma samples were significantly higher ($P<0.05$) than values obtained from conventional anticoagulants.[42]

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Phytochemical Review

1. Nazeema.T.H,Girija.S et al., 2013Evaluated the cytotoxicity of *Jatrophacurcus* and *Jatrophagossypiifolia* on HeLa cell lines and also to isolate and characterize the active principle having highest anticancer activity in the methanolic and ethanoic extract of *Jatrophacurcus* and *Jatrophagossypiifolia*. Crude methanolic and ethanoic fractions of *Jatrophacurcus* and *Jatrophagossypiifolia* stem were examined for their anticancer activity. The anticancer activity was determined for different concentrations of the crude extract on HeLa cancer cell line by (MTT) assay. The extracts were then subjected to high performance liquid chromatography LC Column: Reverse Phase C 18 was used with a detection range of 254 nm.[43]

2. Qiu Sheng – Xiang et al; (2011) evaluated phytochemistry anticancer activity of *J. gossypiifolia* root extract. Phytochemical investigation of the root of *J. gossypiifolia* resulted in the isolation and characterization of one new diterpenoid along with four well-known compounds. The new compound was established by 1D and 2D NMR spectra and x-ray analysis, while spectral (^{1}H, ^{13}C NMR, and ESI-MS) characteristics of the known compounds were compared with reported data. The new compound showed potent proliferation inhibitory activity against A-549 human cancer cell line.[44]

3. Khyade MS et al; (2011) evaluated pharmacognostical and phytochemical on leaves of *Jatropha gossypiifolia L. (Euphorbiaceae*). Macromorphology, microscopy (transverse section, histochemicalcolor reactions and quantitative microscopy) and phytochemical components were studied to establish the salient diagnostic characters. WHO recommends physicochemical determination and phytochemical evaluation for quality control of medicinal plant materials. The various morphological, microscopical, physicochemical standards developed in this study will help for botanical identification and standardization of the drug in crude form.[45]

4. A Falodun, TC Onwudiwe (2011) investigated the seed extract of *Jatropha gossypiifolia* was subjected to phytochemical and antimicrobial investigation using standard screening procedures. The phytochemical studies revealed the presence of some secondary metabolites such as alkaloids, saponins, tannins. There was no activity against the bacteria (Gram positive and negative organisms at 2.5-100mg/ml). The seed extract showed significant antifungal activity. The spectroscopic analysis (1D and 2DNMR) of the colorless oil gave 9-acetoxynerolidol. [46]

3.PLANT PROFILE

Fig 4.*Jatropha gossypiifoliaL.*

Jatropha gossypiifolia

L.Synonym: *Adenoropiumgossypiifolium* (L.) Pohl.

Family: Euphorbiaceae[47]

Vernacular Names

Bengali/Vernacular Name: LalBheranda, Laljeol, Erenda.

Tribal Name: Karachuni (Marma); Kander (Garo).

English Name: Bellyache-bush.

Hindi Name : रतनजोतीRatanjoti

Tamil Name :சிறியஆமணக்குSiriaAmanakku

Other common names

American purging nut, black physic nut, black physicnut, cotton leaf physic nut, cotton-leaf jatropha, cotton-leaf physic nut, purging nut, red fig-nut flower, wild cassava

Habitat

This species is most commonly found in drier tropical environments, but is sometimes also naturalised in sub-tropical and semi-arid regions. It is a weed of degraded pastures, open woodlands, monsoon vine forests, grasslands, riparian vegetation, coastal foreshores, roadsides, disturbed sites, waste areas and old or abandoned gardens.

Distinguishing Features

- Upright shrub or small tree usually growing 0.7-4 m tall.
- Stems are thick and exude a soapy sap when broken.
- Alternately arranged leaves have three or five pointed lobes and are dark reddish-purple when young.
- Small flowers are dark red with yellow centers and are borne in clusters in the upper leaf forks.
- Three-lobed fleshy capsules each contain three seeds.

Chemical Constituents.

- ❖ Leaves contain flavonoids, a saponin, a resin, tannin and triterpenes. They also contain flavonoids, vitexin, isovitexin and apigenin.
- ❖ Roots contain antileukemic and tumour-inhibitor macrocyclicditerpene, jatrophone and jatropholones A and B Flavonoids. Bark contains β-sitosterol.
- ❖ Roots, stems and seeds contain arylnaphthalenelignan and the lignanprasanthaline. Cyclogossine, a cyclic heptapeptide, had been isolated from the latex of the plant.
- ❖ Stem contains a novel lignan, jatrodien[48]
- ❖ Seeds contain fatty oil.

Ethnomedical Uses:

J. gossypiifolia is used for the treatment of various types of disorders in the ayurvedic and folklore system of medicine in Bangladesh.

- The leaves of the plant are traditionally being applied to boils, carbuncles, eczema, itches, and venereal diseases in Latin America and the Caribbean and also used as febrifuge,bark is used as emmenagogue.[49]
- Seeds are emetic, purgative and used for cancer and body pain. The leaves and seeds of *J. gossypiifolia* are considered as a purgative and are widely used to treat obstinate constipation. [50]
- Roots are used to treat leprosy and cancer and stem latex possess coagulant activity. To establish its traditional uses, *J. gossypiifolia* has been investigated for its anti-allergic, molluscicidal, antimicrobial, insect repellent, Larvicidal, anti-feeding, coagulating, and anti-coagulating activities.[51]
- One of the most well-known pharmacological activities of *J. gossypiifolia* is its antineoplastic action, which is frequently associated with the content of lignoids and terpenoids and flavonoids. The ethanoic extract from roots, as well as the isolated diterpenejatrophone, exhibited significant inhibitory activity *in vitro* against cells derived from human carcinoma of the nasopharynx and lymphocytic leukemia P-388 and *in vivo* against four standard animal tumor systems, such as sarcoma 180, Lewis lung carcinoma, P-388 lymphocytic leukemia, and Walker 256 intramuscular carcinosarcoma.[52]

4. RATIONALE FOR SELECTION OF THE PLANT

Jatropha gossypiifolia L. (Euphorbiaceae), widely known as "bellyache bush," is a medicinal plant largely used throughout Africa and America. Several human and veterinary uses in traditional medicine are described for different parts and preparations based on this plant. Previous literature survey have demonstrated ethanomedical uses of different extracts as antimicrobial, anti-inflammatory, antidiarrheal, antihypertensive, and anticancer agents, among others, supporting some of its popular uses.

Anti neoplastic activity

Numerous cancer research studies have been conducted using traditional medicinal plants in an effort to discover new therapeutic agents that lack the toxic side effects associated with current chemotherapeutic agents Therefore the identification of active plant constituent is an essential component of modern phytochemistry and medical effects are not necessarily restricted to a single plant chemical. The biological activity and clinical value of the whole plant, as in medicinal herbalism, is also being pursued. Despite their widespread use, however, less scientific assessment for anticancer effect for the isolated compound from the root extract has been conducted in most cases.[53]

It has been found that the anticancer studies carried out in *Jatropha gossypiifolia* have not made any attempt to characterize and explore the active principles responsible for combating this dreadful disease. Though ample literatures on therapeutic application of these plants are available but data on the proximate compounds responsible for the anticancer activity of these plants are very scarce. Considering their increasing recognition, the present study was undertaken to evaluate the anticancer potential on root extract as well as the isolated compound to determine the inhibition in cell proliferation.[54]

An attempt has been made in the present research, to assess the cytotoxicity on root extracts and fractions of *Jatropha gossypiifolia* on MCF-7 cell line. (Breast Cancer Cell line) and also to isolate the active compound from the potent fraction having highest anticancer activity with the same cell line. The isolated compound is also planned for spectral characterization by hyphenated techniques like FT-IR,^{1}HNMR, GC MS and also subjecting the isolated compound for the *in vitro* anti-cancer activity on MCF-7 Cell line.

5. AIM & OBJECTIVE

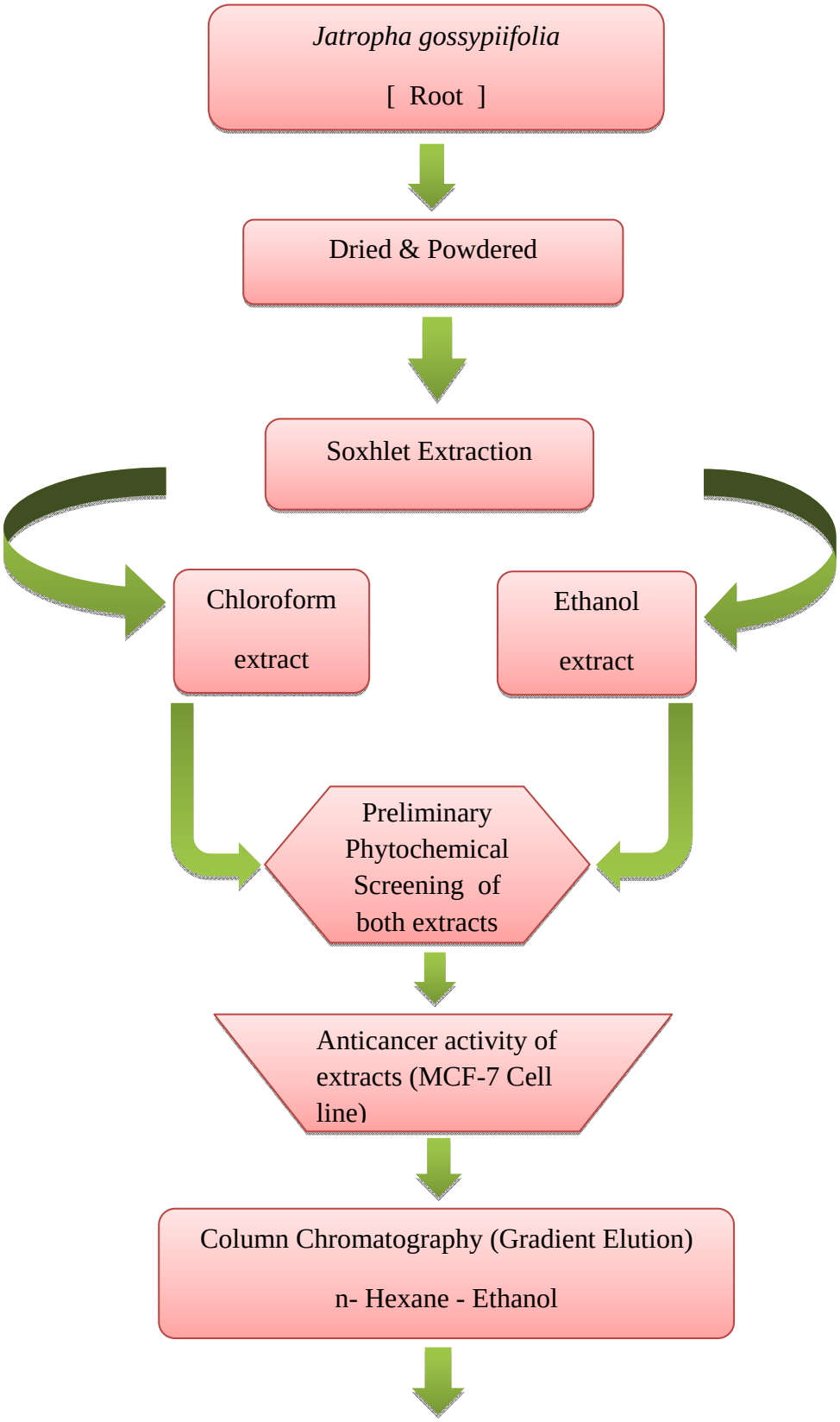
Aim

To evaluate phytochemical and *in vitro* anti-cancer activity on roots of *Jatropha gossypiifolia* Linn

Objective

- ✓ To perform Soxhlet extraction on roots of *Jatropha gossypiifolia*
- ✓ To evaluate preliminary phytochemical screening and *in vitro* anti-cancer activity of the extracts on Breast Cancer line (MCF-7)
- ✓ To subject the active extract for fractionation in column chromatography in solvents from non-polar to polar by gradient elution.(n-hexaneto ethanol)
- ✓ To evaluate the anticancer activity in fractions on Breast Cancer line (MCF-7).
- ✓ To perform Preparative HPLC for the bio active fraction and isolate the pure compound by observing the peak area , height and retention time.
- ✓ To characterize the compound byFT-IR,^{1}HNMR, GCMS and interpret it to determine the structure.
- ✓ To perform the docking studies for the isolated compound on breast cancer target protein human tankyrase 2.
- ✓ To investigate Gene expression of isolated compound by RT PCR method.

6.SCHEME OF WORK



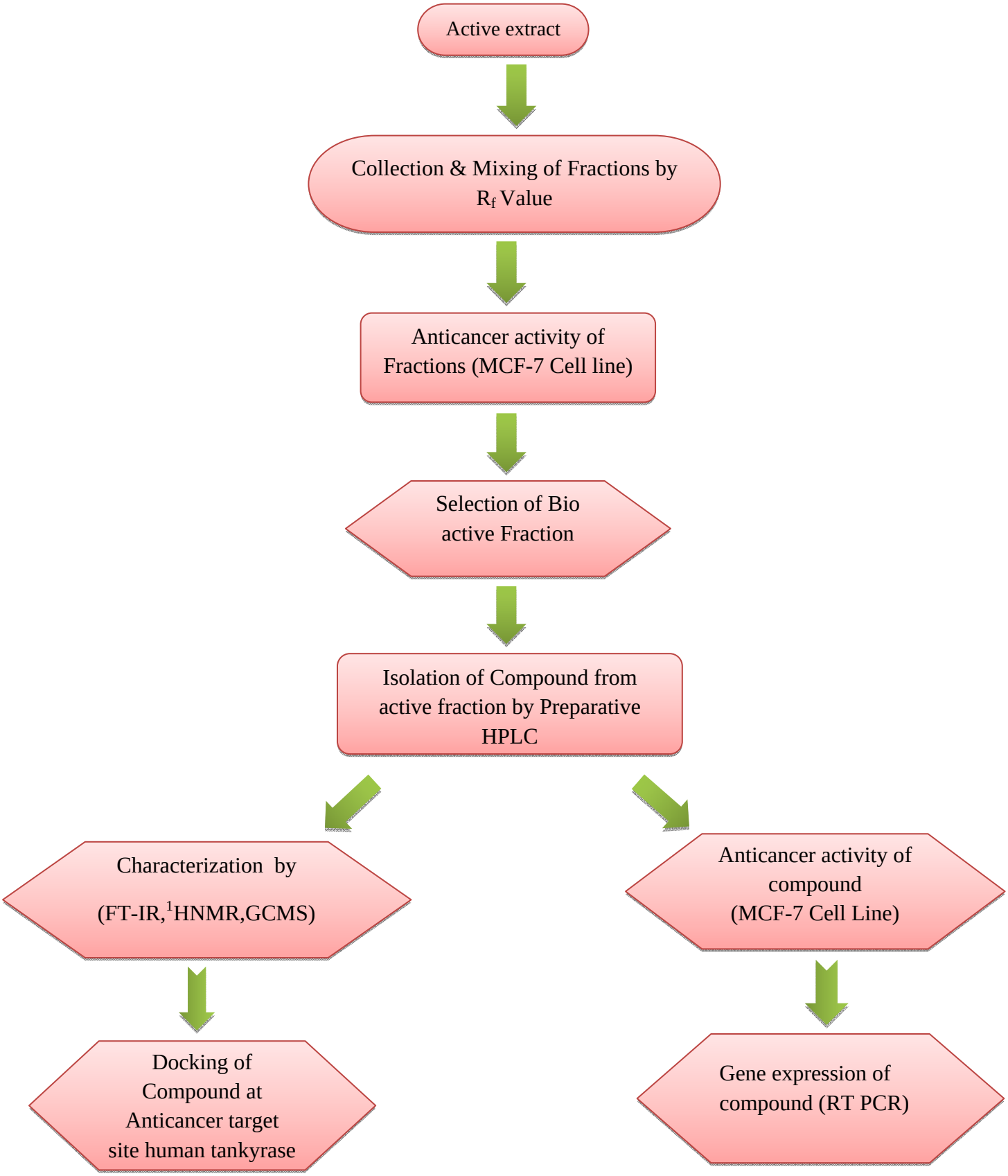
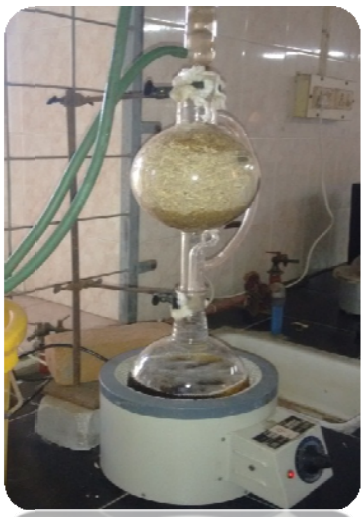
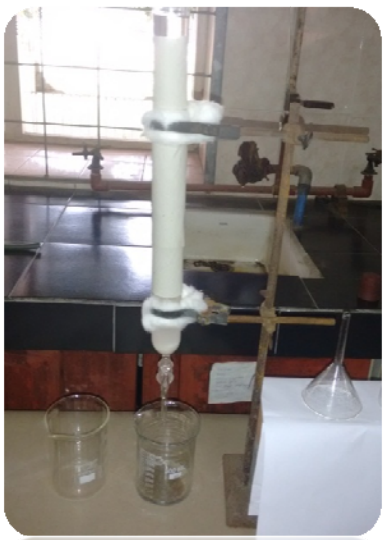

Active extract
Collection & Mixing of Fractions by R_f Value
Anticancer activity of Fractions (MCF-7 Cell line)
Selection of Bio active Fraction
Isolation of Compound from active fraction by Preparative HPLC
Characterization by (FT-IR,^{1}HNMR,GCMS)
Docking of Compound at Anticancer target site human tankyrase
Anticancer activity of compound (MCF-7 Cell Line)
Gene expression of compound (RT PCR)

Fig 5 . Materials used for extraction and separation





7.MATERIALS AND METHODS

Collection of Plant

The roots of *Jatropha gossypiifolia* were collected from Chennai, Tamil Nadu, India. The plant material was identified and authenticated by Botanist Dr.SasikalaEthirajulu, Research officer, CCRAS, Govt.of India, Chennai. The roots of *Jatropha gossypiifolia* were dried under shade, segregated, pulverized by a mechanical grinder and passed through a 40-mesh sieve.

Preparation of extracts

The dried powder of the root was extracted sequentially by hot continuous percolation method bySoxhlet apparatus, using chloroform and ethanol as solvent. The extracts were concentrated by using a rotary evaporator.[55]

PRELIMINARY PHYTOCHEMICAL SCREENING

The extracts obtained were subjected for phytochemical screening using standard procedure. The dried extracts were dissolved in sufficient amounts of respective solvents and tested for various constituents.[56,57]The results of the tests are mentioned in table 2.

Carbohydrates

Molisch's test (General test): To the extract (2 – 3ml) few drops of α naphthol solution was added and then from the sides of the test tube concentrated H_2SO_4 was added drop wise. A violet ring formed at the junction of two liquids indicates the presence of carbohydrates.

Fehling's test: To a mixture of 1ml of Fehling's A and 1ml Fehling's B in a test tube equal volume of extract was added and heated in a boiling water bath for 5 – 10 min. A brick red precipitate indicates the presence of reducing sugars.

Benedict's test: Equal volume of Benedict's reagent was mixed with the extract in the test tube and heated on a boiling water bath. Solution, which appears green, yellow or red, indicated the presence of reducing sugar.

Barfoed's Test: Equal volume of Barfoed's reagent was mixed with extract and heated for 1 -2 min. in boiling water bath and cooled. Red precipitate indicates the presence of monosaccharides.

Aniline acetate test: The extract was boiled in a test tube and the filter paper soaked in aniline acetate was shown in the vapor. Filter paper turns pink confirming the presence of pentose sugars.

Bial'sOrcinols Test: To the boiling Bial's reagent few drops of extract was added. Appearance of green or purple color indicates the presence of pentose sugars.

Seliwinoff's Test: 3ml of seliwinoff's reagent was heated with 1ml of extract in water bath for 1 – 2min. Formation of red color indicates the presence of hexose sugars (fructose).

Tollen'sPhloroglucinoltest: 2.5ml of Con. HCl was mixed with 4ml of 0.5% Phloroglucinol and 1 – 2ml of extract. Appearance of yellow to red color on heating shows the presence of hexose sugar (galactose).

Extract on treatment with Fehling's and Benedict's reagent and indicates the presences or absence of non-reducing sugars. Positive response towards reagent confirms the presence of non-reducing sugars and vice versa.

Iodine Test: 3 ml of extract was mixed with few drops of Iodine solution. Appearance of blue colour indicates the presence of starch.

Test for gums: Hydrolyzed extract with dilute HCl was mixed with Fehling's or Benedicts reagents. Appearance of red color shows the presence of gums.

Test for mucilage: Powdered material was treated with ruthenium red. A formation of red color indicates the presence of mucilage.

Proteins

Biuret Test:3ml of extract was added with 4% NaOH and few drops of 1% $CuS0_4$ solution. Violet or pink colour formation indicates the presence of proteins.

Millon's Test: The extract was mixed with Millon's reagent. Formation of white precipitate at first which later turns to brick red on warming shows the presence of proteins.

Ninhydrin test: 3ml of extract was heated with 3 drops of ninhydrin solution in boiling water bath for 10 min. Purple or bluish color appearance shows the presence of amino acids.

Fats and Oils.

Solubility Test: The solubility of extract was checked in various solvents. Oils are soluble in ether, chloroform and benzene and insoluble in alcohol and water.

Saponification test: The concentrated extract was boiled with 25mlof 10% NaOH in boiling water bath for 30min and cooled. An excess of Na_2S0_4 solution was added. Soap forms and rises to the top. filter. To the filtrate add H_2S0_4. Evaporate. Collect the residue, it contain glycerol. Dissolve the residue in ethanol. With Ethanol solution, the following test was performed.

To the Ethanol solution few drops of $KHSO_4$, was added and heated. Pungent odour of acrylic aldehyde indicates the presence of fats.

To the ethanol solution few drops of $CuS0_4$ and NaOH solution were added. Appearance of clear blue solution shows the presence of fats.

Steroids

Salkowski reaction: 2 ml of extract was mixed with 2 ml of chloroform and 2 ml of conc H_2S0_4 through the sides of the test tube. After shaking, appearance of red colour in chloroform layer and green fluorescence in the acid layer shows the presence of steroid.

Libermann – Burchard reaction (LB test): 2ml of the extract with chloroform was mixed with 1 – 2ml acetic anhydride and 2 drops of conc. H_2S0_4 through the sides of the test tube. Appearance of red colour, then blue and finally green colour indicates the presence of steroid.

Libermann reaction: 3ml of the extract was mixed with 3ml acetic anhydride and heated gently and cooled. Few drops of Con. H_2S0_4 were added through the sides of the test tube. Appearance of blue colour indicates the presence of steroids.

Volatile oils

Hydro & aqueous distillation of the drug material shows the presence of volatile oil and following test confirms the volatile oil. A drop of oil was placed on a filter paper. If the filter paper is not permanently stained with oil, presence of volatile oil is confirmed.

Solubility test: The solubility of oil in 90% alcohol shows the presence of volatile oils.

Glycosides

Cardiac glycosides

Baljet Test: Sodium picrate is added to the test drug. Appearance of yellow to orange colour shows the presence of cardiac glycosides.

Legal test: To the extract 1ml of pyridine and 1ml sodium nitroprusside was added. Appearance of pink to red colour indicates the presence of cardiac glycosides.

Keller Kiliani Test: To 2ml of extract, 0.5ml of glacial acetic acid, one drop of $FeCl_3$and conc H_2S0_4 were added. Appearance of reddish brown layer at the junction of two liquids indicates the presence of digitoxose sugar.

Anthracene glycosides

Borntrager's test: 2ml of extract was boiled with 0.5ml of dil. H_2SO_4 and filtered. To the cold filtrate, equal volume of benzene or chloroform was added and the organic layer was separated and treated with few drops of ammonia solution. Appearance of pink or red shows the presence of anthraquinone glycosides.

Modified Borntrager's test: 2 ml of extract was heated with few drops of dil. H_2SO_4 , $FeCl_3$, dil. HCl for 5 min on water bath. After cooling few ml of organic solvent was added

and shaken. The organic layer was separated and equal volume of dil. Ammonia solution was added. Appearance of pink or red colour shows the presence of C glycosides.

Saponin glycosides

Foam test: The drug powder or the extract was shaken vigorously with water. A persistent foam indicates the presence of saponin glycosides.

Haemolytic test: To the extract or the drug powder, one drop of blood was added. Appearance of haemolytic zone indicates the presence of saponin glycosides.

Cyanogenetic glycosides

Grignard reaction or sodium picrate test: A filter paper strip was soaked in 10% acetic acid, then in 10% sodium carbonate and dried. The moistened powdered drug was placed in a conical flask and closed with cork placing the filter paper strip below the cork. Appearance of brick red or maroon color in the filter paper indicates the presence of cyanogenetic glycosides.

To the dry powder or the extract, a drop of 3% mercurous nitrate solution was added. Formation of metallic mercury indicates the presence of cyanogenetic glycosides.

Coumarin glycosides

The extracts made alkaline with dil. NaOH shows green or blue fluorescence indicating the presence of coumarin glycosides.

Phenolic compounds

To the extract, a few drops of $FeCl_3$ solution were added. Appearance of deep blue to black color shows the presence of phenolic compounds.

The extract was treated with dil. Iodine solution. Formation of transient red colour indicates the presence of phenolic compounds.

To the extract, a few drops of lead acetate solution were added. Formation of white precipitate shows the presence of phenolic compounds.

The extract was treated with dil NH_4OH and potassium ferricyanide solution. Appearance of red colour shows the presence of phenolic compounds.

The extract was mixed with dil. HNO_3 and formation of red to yellow colour indicates the presence of phenolic compounds.

Flavonoids

Shinoda test: The extract was treated with 0.5g of magnesium turnings and few drops of conc. HCl from the sides of the test tube. Appearance of pink colour shows the presence of flavonoids.

To the extract, few drops of lead acetate solution were added. Formation of yellow precipitate indicates the presence of flavonoid.

The extract was mixed with excess amount of sodium hydroxide solution. Appearance of yellow coloration indicates the presence of flavonoid.

Alkaloids

The extract was acidified with dil. HCl and filtered. The following tests were performed using the filtrate.

Dragendroff's test: To the filtrate, a few drops of Dragendroff's reagent were added. Formation of orange brown precipitate shows the presence of alkaloids.

Mayer's Test: The filtrate was treated with few drops of Mayer's reagent. Formation of cream coloured precipitate indicates the presence of alkaloids.

Hager's test: The filtrate was treated with few drops of Hager's reagent. Appearance of yellow precipitate shows the presence of alkaloids.

8. BIOACTIVE GUIDED ISOLATION

In vitro anticancer activity of Extracts

MTT assay[58,59]

In vitro cytotoxic effect on extractsof *Jatropha gossypiifolia* was evaluated by using MTT assay In brief, the trypsinized cells from T-25 flask were seeded in each well of 96-well flat-bottomed tissue culture plate at a density of 5x103 cells/well in growth medium and cultured at 37°C in 5% CO_2 to adhere. After 48hr incubation, the supernatant was discarded and the cells were pretreated with growth medium and were subsequently mixed with different concentrations of test compounds (0, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5mg/ml) in triplicates to achieve a final volume of 100µl and then cultured for 48hr. The extract and fractions was prepared as 1.0 mg/ml concentration stock solutions in PBS. Culture medium and solvent are used as controls. Each well then received 5µl of fresh MTT (0.5mg/ml in PBS) followed by incubation for 2hr at 37°C. The supernatant growth medium was re-moved from the wells and replaced with 100µl of DMSO to solubilize the colored formazan product. After 30 min incubation, the absorbance (OD) of the culture plate was read at a wavelength of 572 nm on an ELISA reader, Anthos 2020 spectrophotometer. The percentage cell growth is determined and EC_{50} is determined.The term half maximal effective concentration (EC_{50}) refers to the concentration of a drug, antibody or toxicant which induces a response halfway between the baseline and maximum after a specified exposure time. It is commonly used as a measure of drug's potency.

ISOLATION BY COLUMN CHROMATOGRAPHY

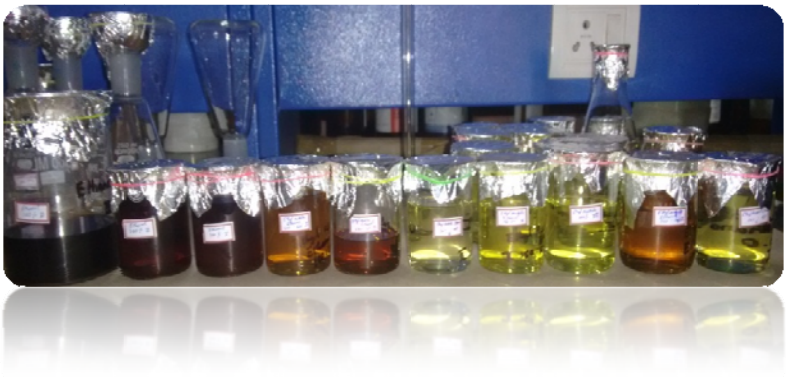
Ethanol extract was subjected for column chromatographic isolation and wet packing method was followed. Initially 3/4th of the column is filled with hexane and then silica gel (100 – 200 mesh size) is added slowly to ensure uniform packing. 20gms of ethanol extract was chromatographed over a column of 400gms silica gel by gradient elution. The column was developed by elution with hexane (100%) followed by hexane: pet ether (160:40, 150:50, 100:100), pet ether (100%) and pet ether: chloroform (160:40, 150:50, 100:100), chloroform (100%) and chloroform: ethyl acetate (160:40, 150:50, 100:100), ethyl acetate (100%) and ethyl acetate : ethanol (160:40, 160:40, 150:50, 100:100) and finally ethanol 100%. The fractions were collected 200ml each. The yellowish green fractions were started eluting from chloroform (100%) and chloroform: ethyl acetate (160:40, 150:50, 100:100, and ethyl acetate

(100%) The brownish colour fractions were eluted from ethyl acetate: ethanol (160:40, 150:50, and 100:100) and finally ethanol 100%.

15 fractions were collected in 200ml each.TLC was determined for the above fractions. The fractions with a same R_f value were mixed together to finally contain 4 fractions i.e (chloroform 100%), (chloroform: ethyl acetate), (ethyl acetate: ethanol), (ethanol 100%).

All these fractions are subjected to anticancer activity on MCF-7 cell line to determine the active and potent fraction against MCF-7 cells.

Fig 6. Fractions collected



Purification of active fraction by Preparative HPLC

Importance of Preparative HPLC

The term preparative HPLC is usually associated with large columns and high flow rates. However, it is not the size of the instrumentation or the amount of mobile phase pumped through the system that determines a preparative HPLC experiment, but rather the objective of the separation. The objective of an analytical HPLC run is the qualitative and quantitative determination of a compound. For a preparative HPLC run it is the isolation and purification of a valuable product. Since preparative HPLC is a rather expensive technique, compared to traditional purification methods such as distillation, crystallization or extraction, it had been used only for rare or expensive products. With increasing demand for production of highly pure compounds in varying amounts for activity, toxicology and pharmaceutical screenings the field of operation for preparative HPLC is changing.

Preparative HPLC is used for the isolation and purification of valuable products in the chemical and Pharmaceutical industry as well as in biotechnology and biochemistry. For identification and structure elucidation of unknown compounds in synthesis or natural product chemistry it is necessary to obtain pure compounds in amounts ranging from one to a few milligrams.[60]

Procedure

Analytical HPLC

Analytical HPLC was performed with a Cosmosil C-18 column (15.0mm×4.6 mm, 5µm) using a Gilson Model 576 pump. The mobile phase was Methanol: water = 90:10 containing 0.1% TFA. Before delivering into the system, it was filtered through 0.45µm, and degassed using a vacuum. The analysis was carried out under isocratic conditions using a flow rate of 1.0 mL/min at room temperature (25ºC). Chromatograms were recorded using a Shimadzu SPD-10A vp UV-VIS detector, with monitoring at 237nm. The injection volume was 20µL for analytical-size injections.

Preparative HPLC

Preparative HPLC was performed with a Gilson Model 576 pump and column (Cosmosil C18-AR-II, 20 mm × 250 mm, 5µm) using Methanol: Water = 90:10 containing 0.1% TFA as mobile phase at a flow rate of 5.4mL /min; UV-Vis detector, monitoring on 237nm. Chromatogram of isolated compound was concentrated by rotary evaporator and analyzed by analytical HPLC to check the purity prior to characterization.[60]

9. CHARACTERIZATION

Instruments used for characterization:

FTIR (Fourier Transform Infra-red Spectroscopy) is a sensitive technique particularly for identifying organic chemicals in a whole range of applications although it can also characterize some inorganics. Examples include paints, adhesives, resins, polymers, coatings and drugs. It is a particularly useful tool in isolating and characterizing organic contamination.

NMR or nuclear magnetic resonance spectroscopy is a technique used to determine a compound's unique structure. It identifies the carbon-hydrogen framework of an organic compound. Using this method and other instrumental methods including infrared and mass spectrometry, scientists are able to determine the entire structure of a molecule

GCMS:

Principle of GC MS a combination of two different analytical techniques, Gas chromatography GC) and Mass Spectrometry (MS), is used to analyze complex organic and biochemical mixtures [61] The GC - MS instrument consists of two main components. The gas chromatography portion separates different compounds in the sample into pulses of pure chemicals based on their volatility Oregon State University, 2012)by flowing an inert gas (mobile phase), which carries the sample, through a stationary phase fixed in the column. Spectra of compounds are collected as they exit a chromatographic column by the mass spectrometer, which identifies and quantifies the chemicals according their mass-to-charge ratio (m/z). These spectra can then be stored on the computer and analyze.

10. DOCKING OF ISOLATED COMPOUND

Procedure:

Glide Docking[62]

The process of docking a set of compounds (ligands) from a file to a PDB target that has a co crystallized ligand. The process involves preparing the protein, preparing the ligands, setting up a grid, and docking the compounds.Preprocessing is necessary because modeling requires 3D, all-atom structures with hydrogens.

Preparing the Protein

1. Choose thetasks Protein Preparation or Applications and then Protein Preparation Wizard.
2. In the Import and Process tab, enter the PDB ID into the PDB text box, and click Import.
3. Choose options for preprocessing the protein structure (other than the defaults).

• If waters in the active site is not needed , enter 0 in the Delete waters beyond text box.

• If needed convert selenium atoms to sulfur, select Convert selenomethionines to methionines.

4. Then Click Preprocess.
5. Address any problems reported in the Protein Preparation in the Problems dialog box.

• If the problems are far from the active site, select the Fill in options and Cap termini, and click Preprocess again.

• If the problems are in or near the active site, run a Prime Refinement for the problem side chains and loops.

6. Delete unwanted parts of the system (chains, solvent molecules, etc.) in the Review and Modify tab.
7. Optimize the H-bond network in the Refine tab.
8. Click Minimize to run a restrained minimization on the structure.

Preparing the Ligands

1. Choose Tasks in that Ligand Preparation or Applications and then LigPrep.
2. Set Use structures from to File, and then click Browse to find and select your ligand file.
3. If Epik is installed, under Ionization select Using: Epik.
4. If the protein has a metal in the binding site, select Add metal binding states (requires Epik).
5. Click Run to run the job. If the ligand file is large, distribute the job over multiple processors if possible.

Generating the Receptor Grid

1. Choose Tasks → Docking → Grid Generation or Applications → Glide → Receptor Grid Generation.
2. Display the prepared receptor in the Workspace.
3. Pick the ligand to define the grid center.
4. Adjust the size of the active site in the Site tab to accommodate larger ligands, if necessary.
5. Add any constraints in the Constraints tab.
6. Pick any rotatable hydroxyl or thiol groups in the active site if such groups could rotate during docking.
7. Add any excluded volumes to exclude atoms from regions other than the receptor.
8. Start the grid generation job.

Docking the Ligands

1. Choose Tasks → Docking → Glide Docking or Applications → Glide → Ligand Docking.
2. Specify the receptor grid to use.
3. Select the docking precision:

• HTVS for initial screen of millions of compounds (shows limited conformational search but fast)
• SP for thousands of compounds (better coverage of conformational space)
• XP for tens or hundreds of compounds (high accuracy on docked poses)

4. If XP is selected, select Write XP descriptor information if you want to visualize interaction terms.

5. Select Add Epik state penalties to docking score, if Epik was used in ligand preparation (especially for metalloproteins).
6. Specify the ligand file to use, in the Ligands tab.
7. If want to set up constraints to a reference ligand core or calculate RMSD to this core, you can do this in the Core tab.
8. Select the receptor constraints you want to use in the Constraints tab, and supply any required information.
9. If the ligands are very flexible, apply constraints on ligand torsions in the Torsional Constraints tab, to reduce the torsional degrees of freedom.
10. Set the number of poses per ligand and total number of poses in the Output tab.
11. Use post-docking minimization to improve pose geometries if needed.
12. Select Write per-residue interaction scores for residues within N Å of grid center if you want to examine interactions of ligand poses with the receptor, and set the cutoff distance.
13. Run the job. If the ligand file is large, distribute the job over multiple processors if possible.

Examining Poses

1. Import the pose file *jobname*_pv.mae into Maestro. Ensure that the option For pose viewer files, turn on pose viewing is selected.
2. In the View Poses panel, turn on display of H-bonds and contacts. If you wrote out per-residue interaction scores, select Display in the Per-residue interactions section.
3. Use the LEFT ARROW and RIGHT ARROW keys to step through the poses, and examine their interactions with the receptor.
4. After finishing examining poses, right-click on the receptor entry in the Project Table and choose Unfix to exit the pose viewing mode.

11. *IN VITRO* ANTICANCER ACTIVITY OF ISOLATED COMPOUND

In vitro cytotoxic effect on extractsof*Jatrophagossypiifolia* was evaluated by using MTT assay against MCF -7 cell line. MCF cells were purchased from NCCS Pune. In brief, the trypsinized cells from T-25 flask were seeded in each well of 96-well flat-bottomed tissue culture plate at a density of 5x103 cells/well in growth medium and cultured at 37°C in 5% CO_2 to adhere. After 48hr incubation, the supernatant was discarded and the cells were pretreated with growth medium and were subsequently mixed with different concentrations of test compounds (0.1µg, 1µg, 10µg and 100µg) in triplicates to achieve a final volume of 100µl and then cultured for 48hr. The extract and fractions was prepared as 1.0 mg/ml concentration stock solutions in PBS. Culture medium and solvent are used as controls. Each well then received 5µl of fresh MTT (0.5mg/ml in PBS) followed by incubation for 2hr at 37°C. The supernatant growth medium was re-moved from the wells and replaced with 100µl of DMSO to solubilize the colored formazan product. After 30 min incubation, the absorbance (OD) of the culture plate was read at a wavelength of 572 nm on an ELISA reader, Anthos 2020 Tamoxifen was used as the standard drug and percentage growth and GI_{50} was determined.[58,59, 63]

12. GENE EXPRESSION

Methodology

Cell culture and treatment[64]

1. The MCF-7 breast cancer cell line was purchased from NCCS Pune. The MCF-7 cells were grown in DMEM medium supplemented with 10% fetal bovine serum and antibiotics as mentioned earlier. For the Real time PCR assay, the cells were grown in 25 cm ×25 cm ×25 cm tissue culture flasks containing DMEM medium as culture medium supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin (GIBCO) and grown at 37°C under a humidified atmosphere of 95% air and 5% CO_2. Cells were regularly passaged and maintained before experiments.

2. When the cell density in a culture flask reached 70-80% confluence, they were trypsinized and seeded in 6-well plates at a density of 300000 cells per well in 1000 µL and incubated for 24 hours at CO_2 incubator.

3. Next day, test item was prepared as 100 mg/ml stock by adding directly in to the DMSO. The working stock of 2X (160, 80 and 40, 60 ug/ml) concentration was added to the cells in 1000µL volume and the final concentration range were: 80, 40 20, 100 ug/ml .plate was further incubated for 48 hours in the CO_2 incubator.

RNA isolation -RNeasyMiniprep kit from Qiagen

- The Total RNA is extracted from the treated cells samples
- RNA Later preserved rat liver samples
- Use the protocol from the attached book let page number 38-44.
- ***Determination of RNA purity and concentration***

The final preparation of total RNA is essentially free of DNA and proteins and has a 260/280 ratio 1.9.

High Capacity cDNA Reverse

Table 2.kit components

10× RT Buffer, 1.0 mL	1 tube
10× RT Random Primers	1 tube
25×dNTP Mix (100 mM)	1 tube, 0.2 mL
MultiScribe™ Reverse Transcriptase, 50 U/µL	2 tubes ,0.1ml
RNase Inhibitor, 100 µL	2 tubes

Transcription Kits:

Kit Features Use the kit for:

• Quantitatively converting up to 2 µg (for a 20-µL reaction) of total RNA to cDNA
• Generating single-stranded cDNA suitable for quantitative PCR applications
• Generating single-stranded cDNA suitable for short- or long-term storage

High Capacity cDNA Reverse Transcription Kit, 200 reactions (Part Number: 4374966)

Kit Storage Store all kit components at –15 to –25 °C

Preparing the 2×Reverse Transcription Master Mix:

To prepare the 2×RT master mix (per 20-µL reaction)

Table 3. Volume of Components for preparing Reverse Transcription Master Mix

1. Allow the kit components to thaw on ice.

Component	**Volume/Reaction (µL)**
10✕ RT Buffer	2.0
25✕dNTP Mix (100 mM)	0.8
10✕ RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free H2O	3.2
Template	10
Total Volume (ul)	**20**

2. Referring to the tableabove, calculate the volume of components needed to prepare the required number of reactions.

Note: Prepare the RT master mix on ice.

3. Place the 2✕ RT master mix on ice and mix gently.

Kit with RNase Inhibitor

To prepare the cDNA RT reactions:

1. Pipette 10 µL of 2✕ RT master mix into each well of a 96-well reaction plate or individual tube.

2. Pipette 10 µL of RNA sample into each well, pipetting up and down two times to mix.

3. Seal the plates or tubes with optical Adhesive sheet.

4. Briefly centrifuge the plate or tubes to spin down the contents and to eliminate any air bubbles.

5. Place the plate or tubes on ice until you are ready to load the thermal cycler.

Table 4. Preparation of c DNA RT reaction

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature(°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

Program:

1. Set the reaction volume to **20 µL**.
2. Load the reactions into the thermal cycler.
3. Start the reverse transcription run.

Real Time PCR

- A real-time PCR reaction mixture of 20 µl prepared.
- All reactions were performed in duplicate and Non template control (negative control) contained no template DNA. The PCR conditions were as follows:

Table 5.Contents and volume of Real Time PCR reaction mixture

S. No	**Contents**	**Volume (µl)**
1	SYBR Green mix (2x)	10 µl
2	Forward primer	0.6 µl
3	Reverse primer	0.6 µl
4	Template (c DNA)	1 µl
5	Sterile water	7.8 µl
Total		**20 µl**

End of the rtPCR reaction the samples were stored in -20'C

Table 6. PCR Conditions

Steps	**Temperature (in Deg)**	**Time**
Denaturation	95°C	20 Seconds
Annealing	95°C	03 seconds
Extension	60	30 seconds
Repeat	40 cycles	

13. ACUTE TOXICITY STUDY OF AN ISOLATED COMPOUND

procedure

The acute toxicity studyof an isolated compound was carried out by using the software OSIRIS which is used to determine the toxicity risk such as mutagenic, tumorigenic ,irritant ,reproductive effective , clog p, solubility, molecular weight , TPSA, drug likeness and drug-score for the specific structure of the compound.

14. RESULTS AND DISCUSSION

14.1 Extractive values

Table 7.Extractive values of *Jatropha gossypiifolia*

Solvent	Color	Consistency	% Yield
Chloroform	Yellowish brown	Sticky	17.2
Ethanol	Reddish brown	Sticky	35.5

14.2 Phytochemical screening

Table 8.Phytochemical Screening on root extracts of *Jatropha gossypiifolia*

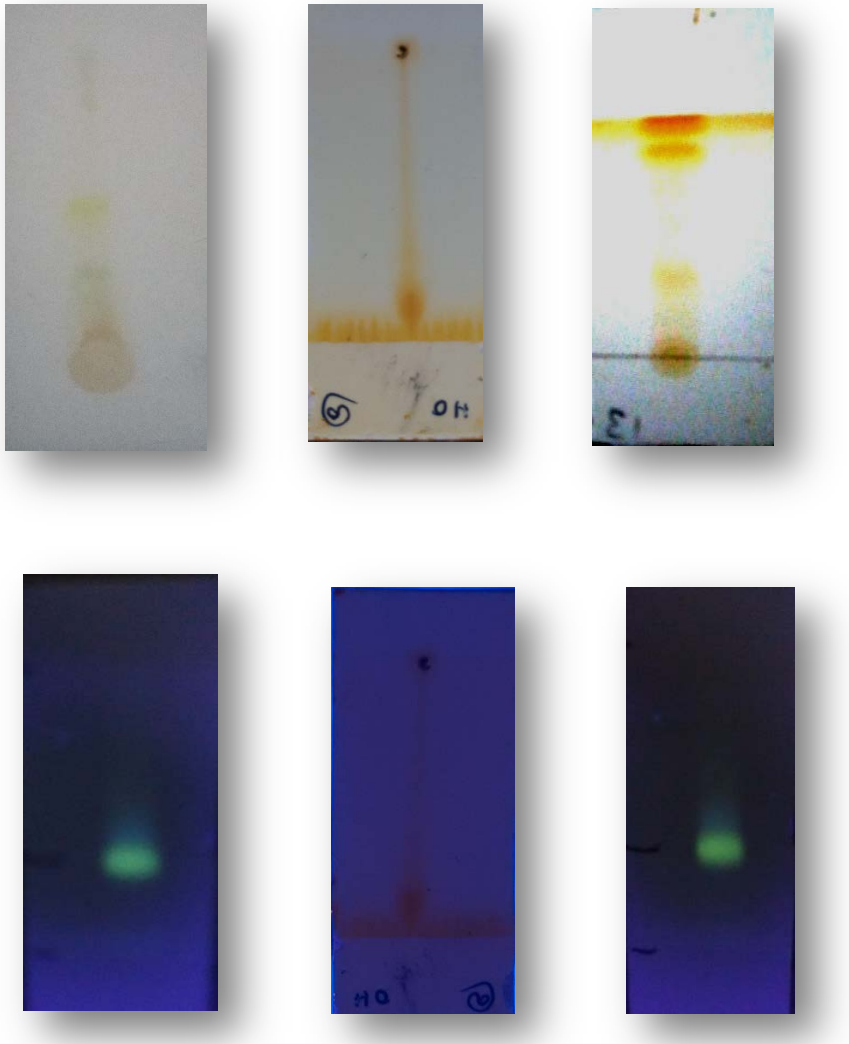
S.N	Name of the test	Chloroform	Ethanol
1.	LibermanBurchard (for terpenes& steroid)	+	-
2.	Salkowski (for steroid &terpenes)	+	-
3.	Fixed oils and fats.	-	-
4.	Mayer's (for alkaloids)	+	-
5.	Molisch (for carbohydrates)	-	-
6.	Fehlings (for carbohydrates)	-	-
7.	Baljet's test for glycosides	+	+
8.	Legal,s test for glycosides	+	+
9.	Test for Phenolics ($FeCl_3$)	-	+
10.	Shinoda (for flavonoids)	-	+

Table 9: Thin Layer Chromatography of Fractions from Column Chromatogrpahy

Fractions	**Solvent system**	**No of Spots**	**R_f value**
Chloroform fraction	Hexane: Chloroform (9:1),(8:2), (7:3)	3	0.7,0.6,0.5
Chloroform:ethyl acetate	Hexane: Ethyl acetate (9:1),(8:2), (7:3) Chloroform:ethylacetate (9:1, (8:2), (7:3)	4	0.6,0.5,0.3,0.2
Ethyl acetate : Ethanol	Chloroform: Methanol (9:1),(8:2), (7:3) Chloroform: ethyl acetate (9:1),(8:2), (7:3)	3	0.7,0.6,0.5
Ethanol (100%)	Chloroform: Methanol (9:1),(8:2), (7:3) Chloroform: ethyl acetate (9:1),(8:2), (7:3)	3	0.7,0.6,0.5

Thin Layer Chromatography

Fig 7 .Thin Layer Chromatography of fractions



15. MTT ASSAY

MTT ASSAY OF EXTRACTS

Table 10. Effect of Cytotoxicity on chloroform extract of *Jatropha gossypiifolia* on MCF-7 Cell Line

Concentration mg/ml	Vehicle		Chloroform extract	
	Absorbance	Percentage growth	Absorbance	Percentage growth
0	1.05800	100.0000	1.05800	100.0000
0.025	1.04560	98.82798	1.05300	99.52741
0.05	1.02370	96.75803	0.83300	78.73346
0.1	099825	94.35255	0.68400	64.65028
0.2	098275	92.88752	0.52203	49.34121
0.3	0.97400	92.06079	0.52012	49.16068
0.4	0.97125	91.80057	0.51475	48.65312
0.5	0.95650	90.40643	0.52475	**49.59830**

EC50: 0.00018 mg/ml

Fig 8. Dose Vs Response curve of Chloroform Extract on MCF-7 Cell line

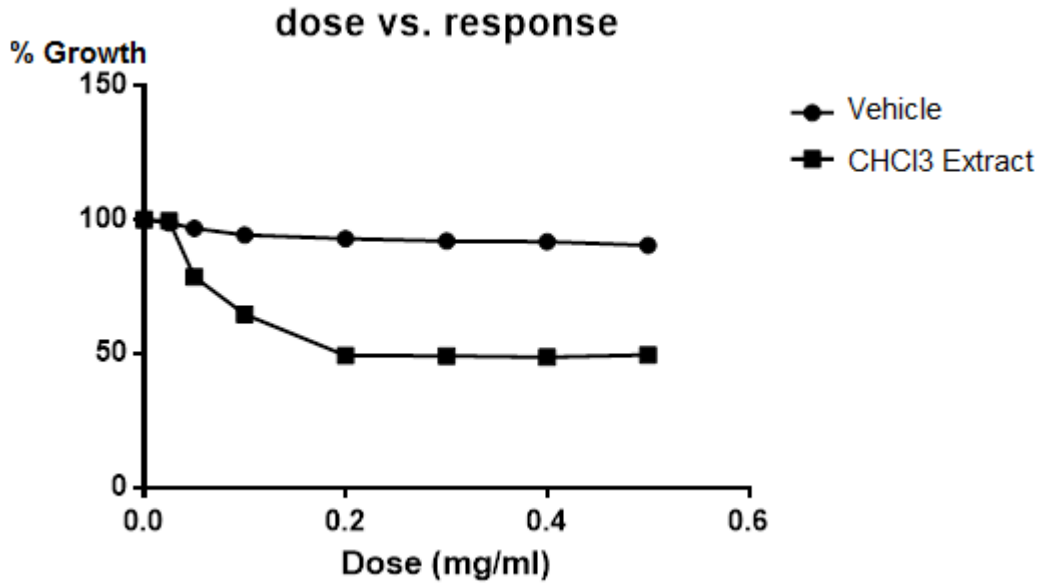


Table 11. Effect of Cytotoxicity on ethanol extract of *Jatropha gossypiifolia* on MCF-7 Cell Line

Concentration mg/ml	**Vehicle**		**Ethanol extract**	
	Absorbance	Percentage growth	Absorbance	**Percentage growth**
0	1.05800	100.0000	1.05800	100.0000
0.025	1.04560	98.82798	0.78225	73.93667
0.05	1.02370	96.75803	0.75222	71.09830
0.1	099825	94.35255	0.70518	66.65217
0.2	098275	92.88752	0.56840	53.72401
0.3	0.97400	92.06079	0.52187	49.32609
0.4	0.97125	91.80057	0.37820	35.74669
0.5	0.95650	90.40643	0.30120	**28.46881**

EC_{50}: 0.00055 mg/ml

Fig 9.Dose vs Response curve of Ethanolic Extract on MCF-7 Cell line

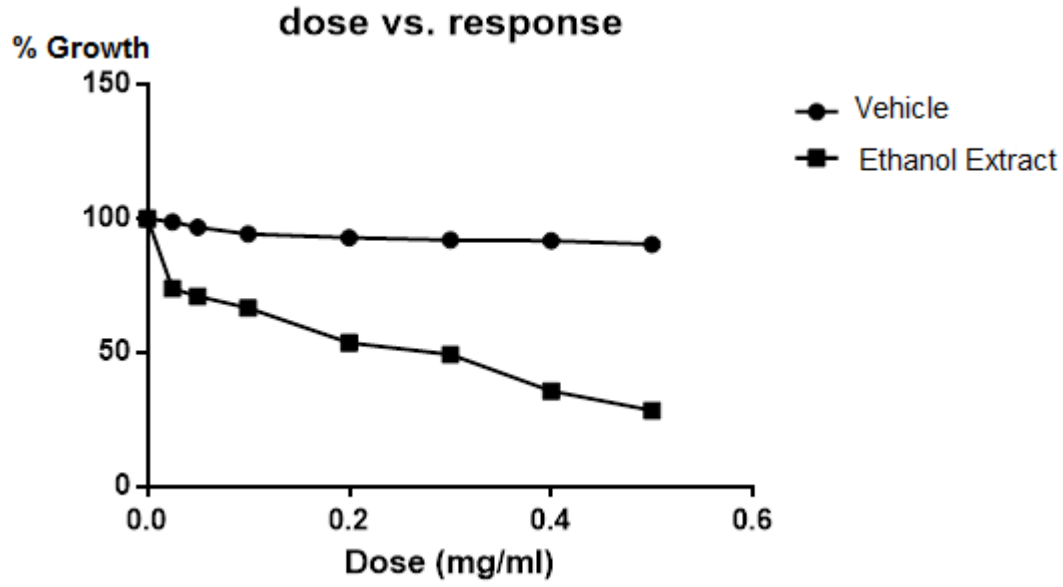
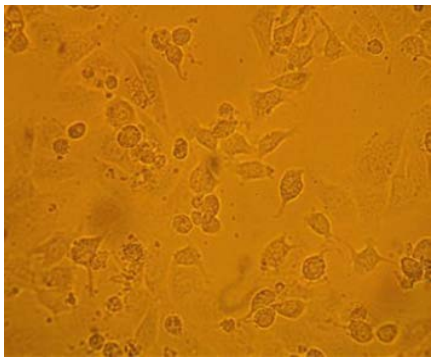
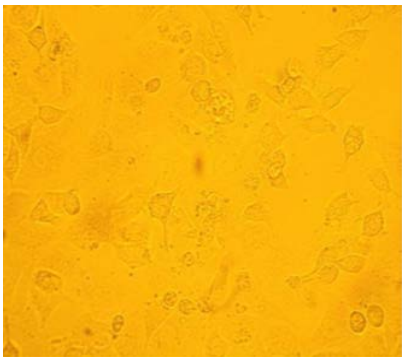


Fig 10. Extracts treated against MCF-7 cell line

Chloroform Extract : (0.5mg/ml)



Ethanol Extract : (0.5mg/ml)



Discussion

Table no 10 & 11 depict the percentage growth of MCF-7 cells in chloroform and ethanol extract treated against MCF-7 cell line was found to be 49.59 and 28.46 respectively. The EC_{50} value of chloroform and ethanol extract was found to be 0.00018 & 0.00055mg/ml respectively. This clearly indicates that ethanol extract have better anticancer activity on MCF-7 cell line than the chloroform extract. As it was found in preliminary phytochemical screening, (table no 8) the ethanol extract shows the presence of flavonoids and phenolic compounds, the better anticancer activity of ethanol extract may be due to the presence of these active compounds.

The percentage growth of MCF-7 cells at 0.5mg /ml of chloroform and ethanol extract 49.59830&28.46881 respectively

MTT ASSAY OF FRACTIONS

Table12. Cytotoxic effect of Chloroform fraction of *Jatropha gossypiifolia* on MCF-7 Cell Line

Concentration mg/ml	**Vehicle**		**Chloroform fraction (Fraction1)**	
	Absorbance	Percentage growth	Absorbance	**Percentage growth**
0	1.05800	100.0000	1.05800	100.0000
0.025	1.04560	98.82798	0.48600	45.93573
0.05	1.02370	96.75803	0.42725	40.38280
0.1	099825	94.35255	0.41275	39.01229
0.2	098275	92.88752	0.28875	27.29206
0.3	0.97400	92.06079	0.28625	27.05577
0.4	0.97125	91.80057	0.26075	24.64556
0.5	0.95650	90.40643	0.25950	**24.52741**

EC_{50}: 0.00082 mg/ml

Fig 11. Dose vs Response curve of Fraction 1 on MCF-7 Cell line

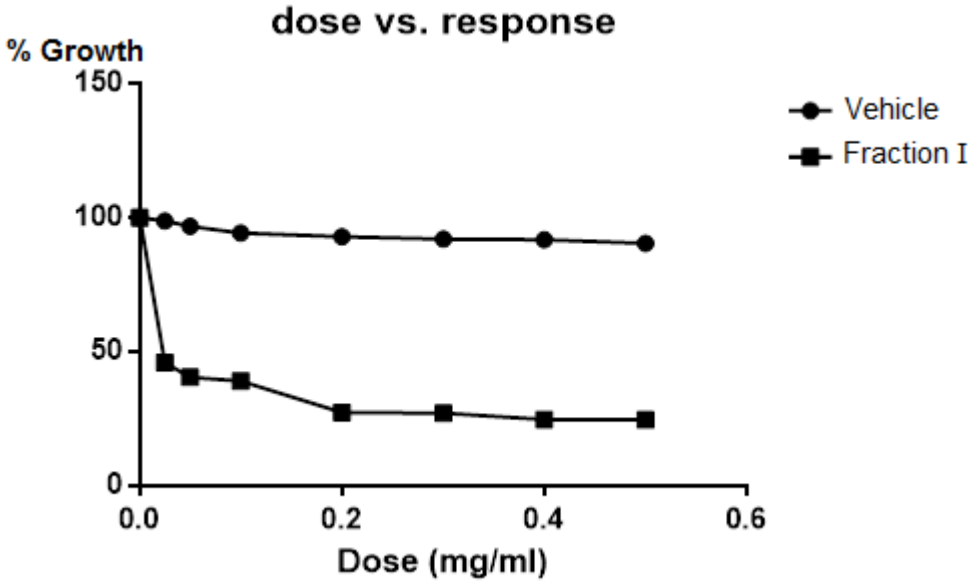


Table 13. Cytotoxic effect of Chloroform: ethyl acetate (fraction 2) of *Jatropha gossypiifolia* on MCF-7 Cell Line

Concentration mg/ml	**Vehicle**		**Chloroform:Ethylcaetate (Fraction2)**	
	Absorbance	Percentage growth	Absorbance	**Percentage growth**
0	1.05800	100.0000	1.05800	100.0000
0.025	1.04560	98.82798	0.99975	94.49433
0.05	1.02370	96.75803	0.94450	89.27221
0.1	099825	94.35255	0.86750	81.99433
0.2	098275	92.88752	0.77575	73.41682
0.3	0.97400	92.06079	0.71909	67.96692
0.4	0.97125	91.80057	0.58050	54.86767
0.5	0.95650	90.40643	0.51982	**49.13233**

EC_{50}: 0.1728 mg/ml

Fig12. Dose response curve for Chloroform: ethyl acetate fraction (2) on MCF-7 cell line.

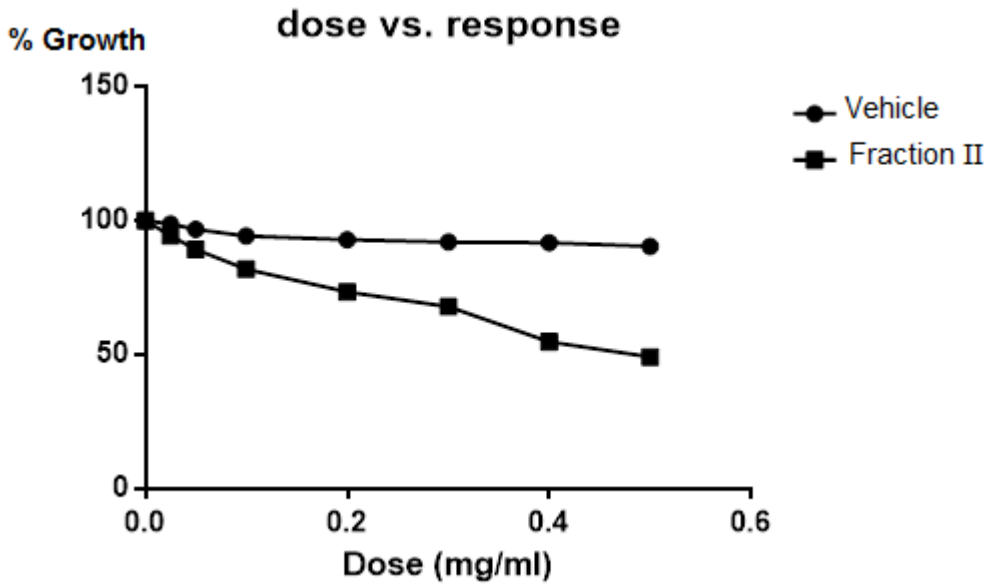


Table14. Effect of Cytotoxicity on ethyl acetate: ethanol (fraction 3) of *Jatropha gossypiifolia* on MCF-7 Cell Line

Concentration mg/ml	**Vehicle**		**Ethyl acetate :ethanol (Fraction3)**	
	Absorbance	Percentage growth	Absorbance	**Percentage growth**
0	1.05800	100.0000	1.05800	100.0000
0.025	1.04560	98.82798	0.95000	89.79206
0.05	1.02370	96.75803	0.78400	74.10208
0.1	099825	94.35255	0.71475	67.55671
0.2	098275	92.88752	0.65200	61.62571
0.3	0.97400	92.06079	0.48500	45.84121
0.4	0.97125	91.80057	0.39375	37.21645
0.5	0.95650	90.40643	0.39050	**36.90926**

EC_{50}: 0.00028 mg/ml

Fig13. Dose response curve for ethyl acetate: ethanol fraction (3) on MCF-7 cell line

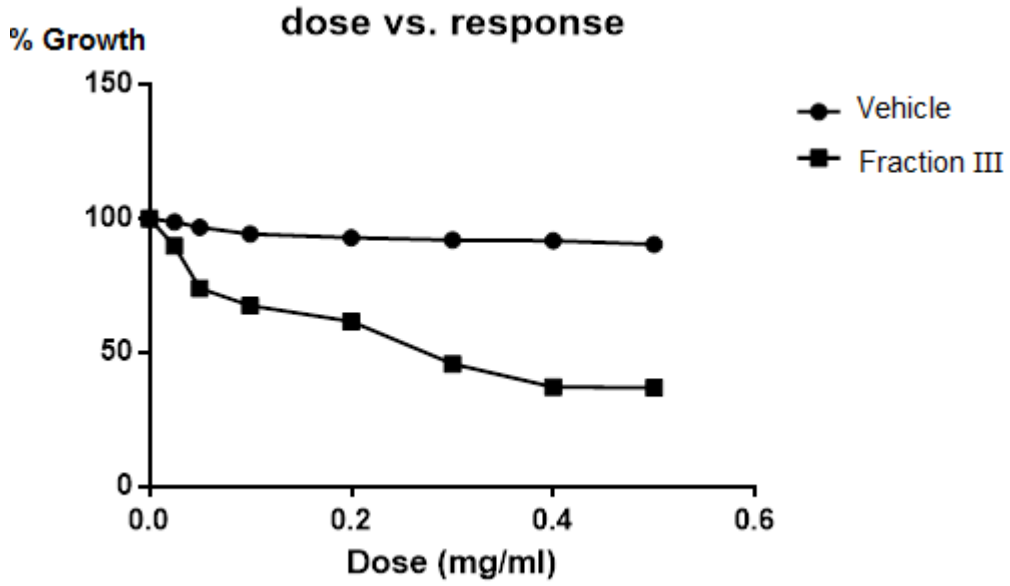
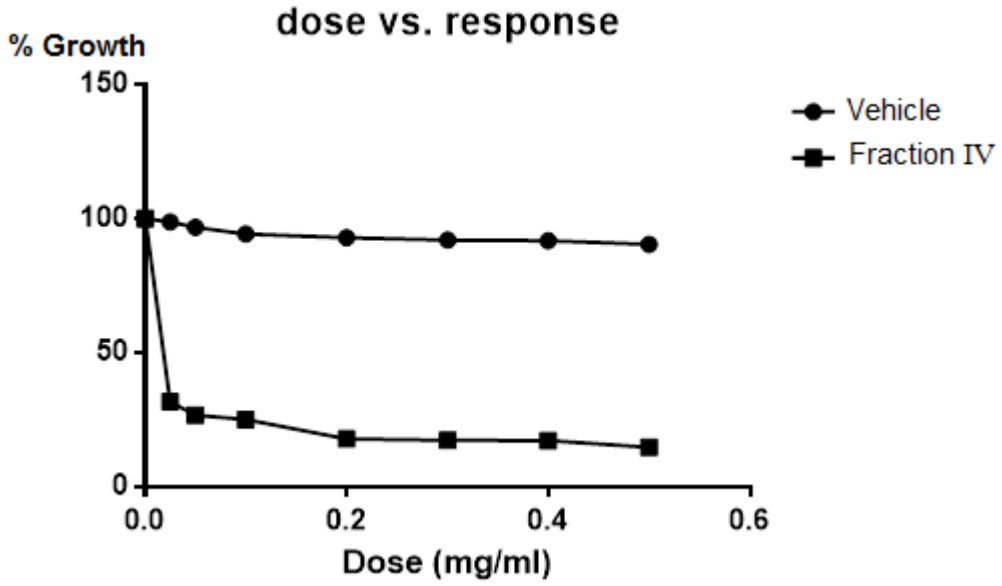


Table 15.Effect of Cytotoxicity on ethanol 100% (fraction 4) of *Jatropha gossypiifolia* on MCF-7 Cell Line

Concentration mg/ml	**Vehicle**		**Ethanol (100%) (Fraction4)**	
	Absorbance	Percentage growth	Absorbance	**Percentage growth**
0	1.05800	100.0000	1.05800	100.0000
0.025	1.04560	98.82798	0.33575	31.73440
0.05	1.02370	96.75803	0.28225	26.67769
0.1	099825	94.35255	0.26450	25.00000
0.2	098275	92.88752	0.18800	17.76938
0.3	0.97400	92.06079	0.18350	17.34405
0.4	0.97125	91.80057	0.18150	17.15501
0.5	0.95650	90.40643	0.15500	**14.65028**

EC_{50}: 0.00087 mg/ml

Fig 14.Dose response curve of ethanol fraction (4) on MCF-7 cell line



DISCUSSION

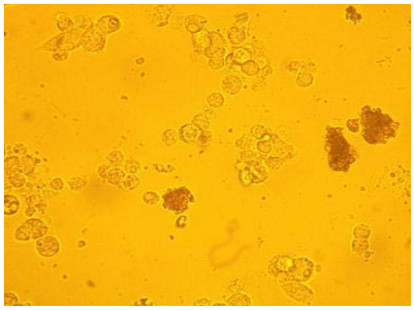
Table 12 to 15& Fig 11 to 14 of dose response curves depict the *in vitro* anticancer activity from fractions (1 to 4) from column chromatography on MCF-7 cell line. The fraction 4 (Ethanol 100%) shows the good anticancer activity compared to other fractions against MCF-7 cell line. The percentage growth of MCF-7 cells in fraction (1 -4) was found to be 24.527, 49.132, 36.909, 14.650 respectively. The EC_{50} value was found to be for fractions (1 to 4) was found to be 0.00082, 0.1728, 0.00028, 0.00087mg/ml respectively.The cytotoxic effect on maximum concentration 0.5mg/ml of fractions treated against MCF – 7 cells was seen in fig 15.

This clearly indicates that fraction (IV) shows good anticancer activity against MCF-7 cell line when compared to other 3 fractions.Hence fraction (1V)is an active fraction.

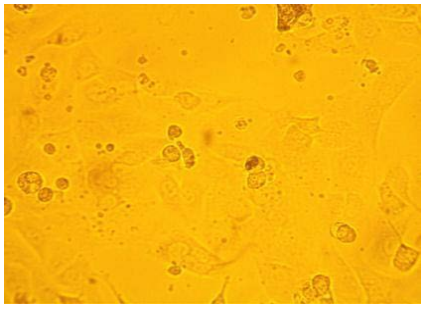
Fig15. Fractions treated against MCF-7 cell line

Chloroform fraction: (0.5mg/ml)Chloroform:ethyl acetate: (0.5mg/ml)

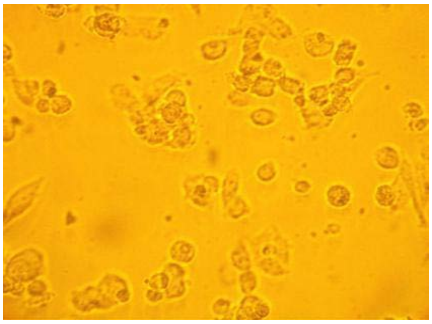
Ethyl acetate: ethanol: (0.5mg/ml)



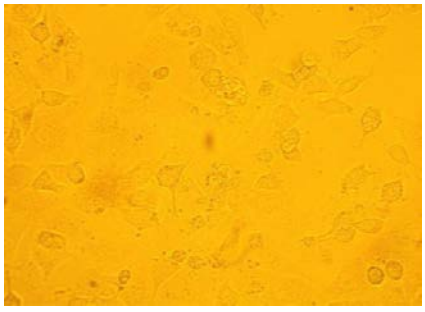
Ethanol 100% : (0.5mg/ml)



Ethyl acetate: ethanol: (0.5mg/ml)



Ethanol 100% : (0.5mg/ml)



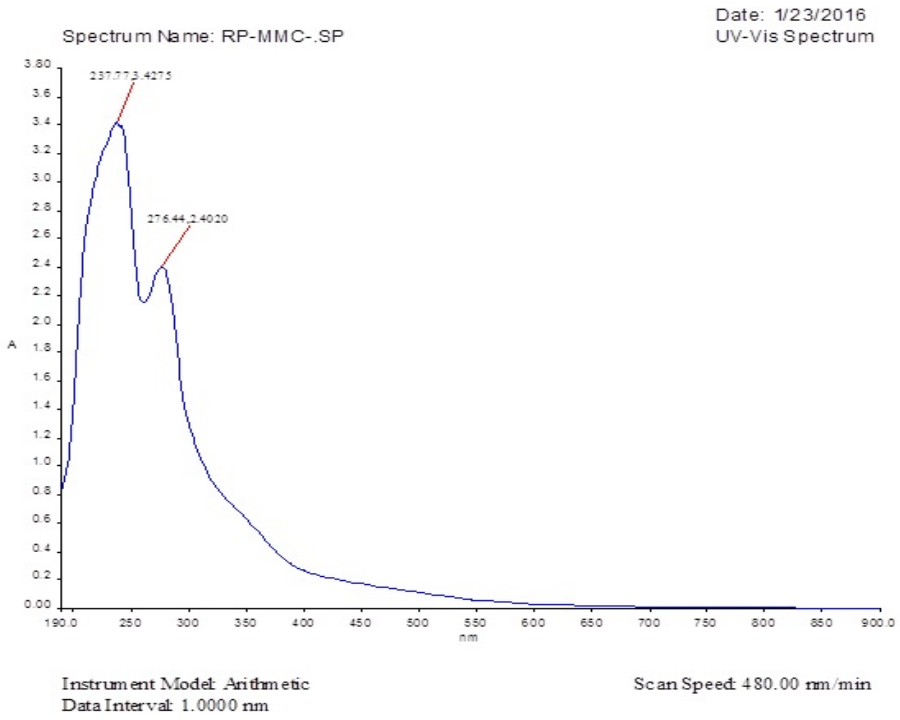
16. RESULTS AND DISCUSSION OF ISOLATION & PURIFICATION

Bio active fraction (Ethanol 100%) was subjected for isolation and purification by Preparative HPLC. In this study, compound isolation is performed effectively by preparative HPLC

UV SPECTROSCOPY

Initially UV Spectrum was carried out to find out the wavelength of maximum absorbance (λ_{max}). The λ_{max} was found to be 237nm.

Fig: 16. UV Visible Spectrum of fraction 1V



Analytical HPLC

The Analytical HPLC was carried out at the same 237nm to find out the number of peaks and also to determine the single peak which has good retention time and higher peak area and height.The analysis was carried out under isocratic conditions using a flow rate of 1.0 mL/min at room temperature (25ºC). In Fig:16**.** Of Analytical HPLC shows that Peak 1 has retention time of 2.645 with height 23359 and area of 89.703. The peak 1 was selected for isolation by Preparative HPLC

Fig 17. HPLC analysis of fraction 1V

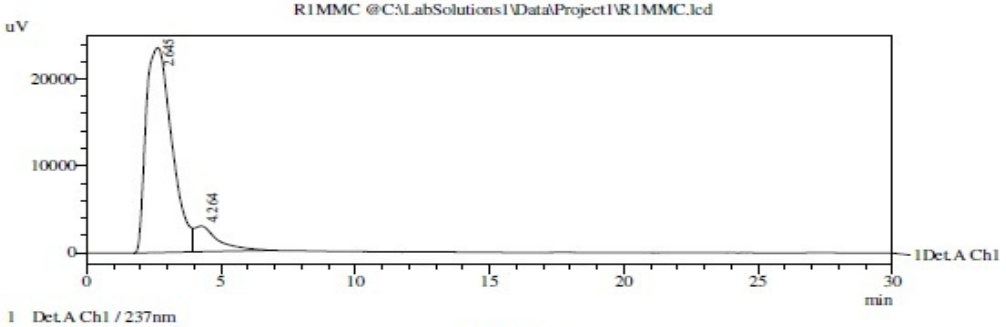
HPLC ANALYSIS REPORT

Sample Information

Acquired by	: Admin
Sample Name	: R1MMC
Sample ID	:
Vail#	:
Injection Volume	: 20 uL
Data Filename	: R1MMC.lcd
Method Filename	: JMC-B-Methd.lcm
Batch Filename	:
Report Filename	: Default.lcr
Date Acquired	: 29-01-2016 PM 01:01:51
Data Processed	: 29-01-2016 PM 01:31:54

Chromatogram

R1MMC @C:\LabSolutions1\Data\Project1\R1MMC.lcd



1 Det.A Ch1 / 237nm

PeakTable

Detector A Ch1 237nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.645	1584076	23559	89.703	88.917
2	4.264	181843	2937	10.297	11.083
Total		1765919	26496	100.000	100.000

Preparative HPLC

The peak 1 was selected for isolation by preparative HPLCusing Methanol: Water = 90:10 containing 0.1% TFA as mobile phase at a flow rate of 5.4mL /min. The isolated peak was collected in 50ml centrifuge tubes. Totally 250ml of fraction has been collected. Hence the single purified compound was separated effectively. Analytical HPLC was also done for the isolated compound to confirm its purity prior to characterization.

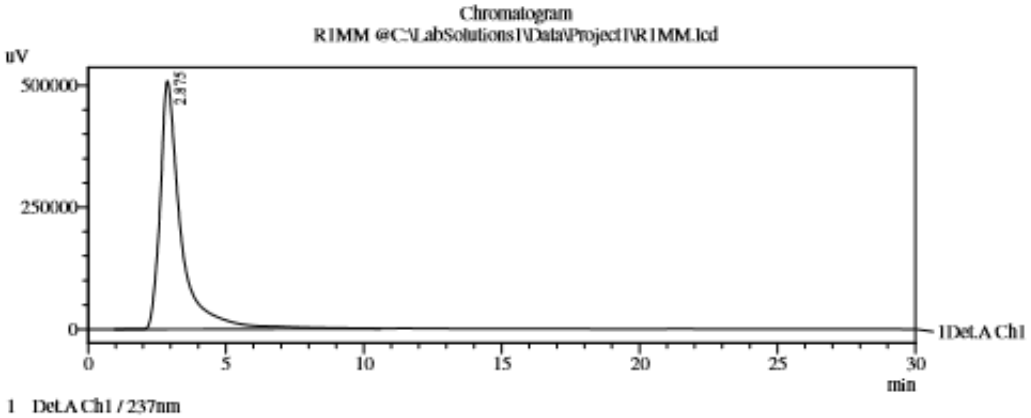
Fig 18 . HPLC Analysis after separation by preparative HPLC

St.Joseph's college (Autonomous), Thiruchirapalli-2.

HPLC ANALYSIS REPORT

Sample Information

Acquired by	: Admin
Sample Name	: R1MM
Sample ID	:
Vail#	:
Injection Volume	: 20 uL
Data Filename	: R1MM.lcd
Method Filename	: MC-Methd.lcm
Batch Filename	:
Report Filename	: Default.lcr
Date Acquired	: 10-03-2016 AM 10:15:00
Data Processed	: 10-03-2016 AM 10:46:01



1 Det.A Ch1 / 237nm

PeakTable

Detector A Ch1 237nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.875	26689386	508506	100.000	100.000
Total		26689386	508506	100.000	100.000

Sodium hydroxide test:

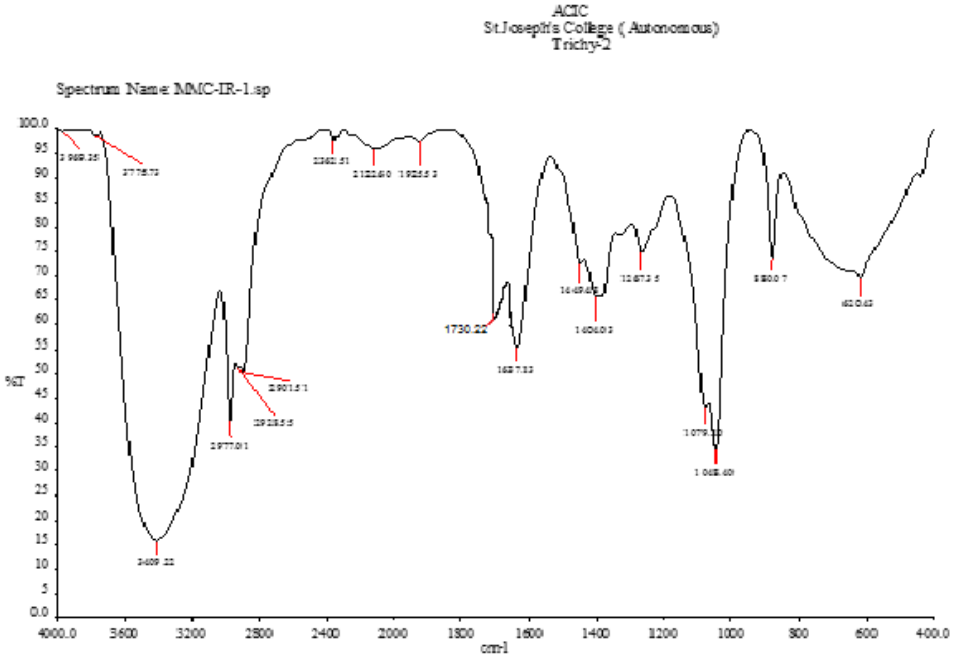
The little amount of isolated compound is dissolved in water, warmed and filtered. 10% aqueous sodium hydroxide is added to 2 ml of this solution. This produces a yellow coloration. A change in color from yellow to colorless on addition of dilute hydrochloric acid is an indication for the presence of flavonoids.[65]

The isolated compound was subjected to qualitative analysis for the test of flavonoid and phenolic compounds. The compound gives the positive test for flavonoid.

17. RESULTS OF CHARACTERIZATION OF ISOLATED COMPOUND

FT- IR spectra of isolated compound.

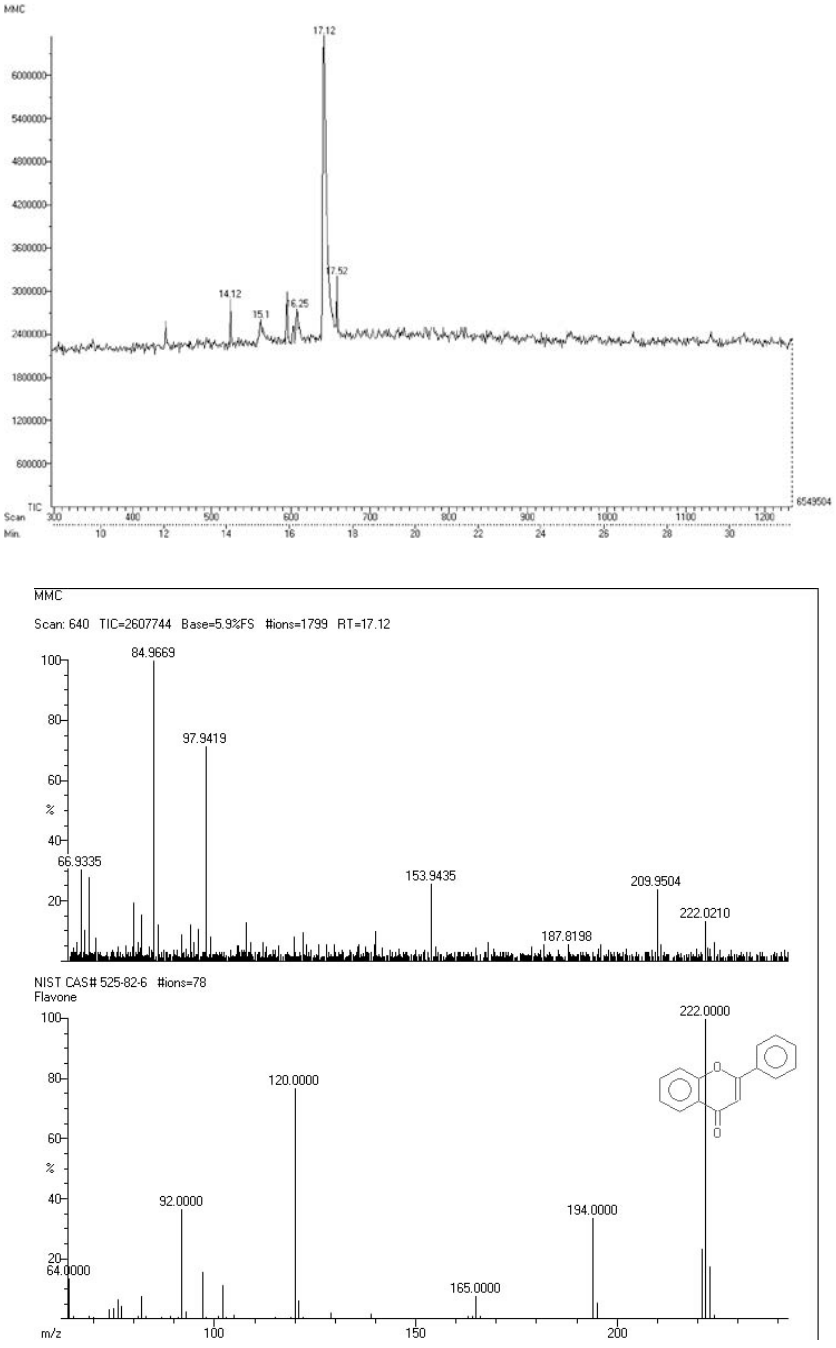
Fig 19. FT- IR Spectrum of an Isolated Compound



2977 cm^{-1} (Aromatic CH stretching), 1730 cm^{-1},C=O (Carbonyl group of Chromene ring), 1637cm^{-1}(Aromatic C=C).

GC MS Spectrum of Isolated compound

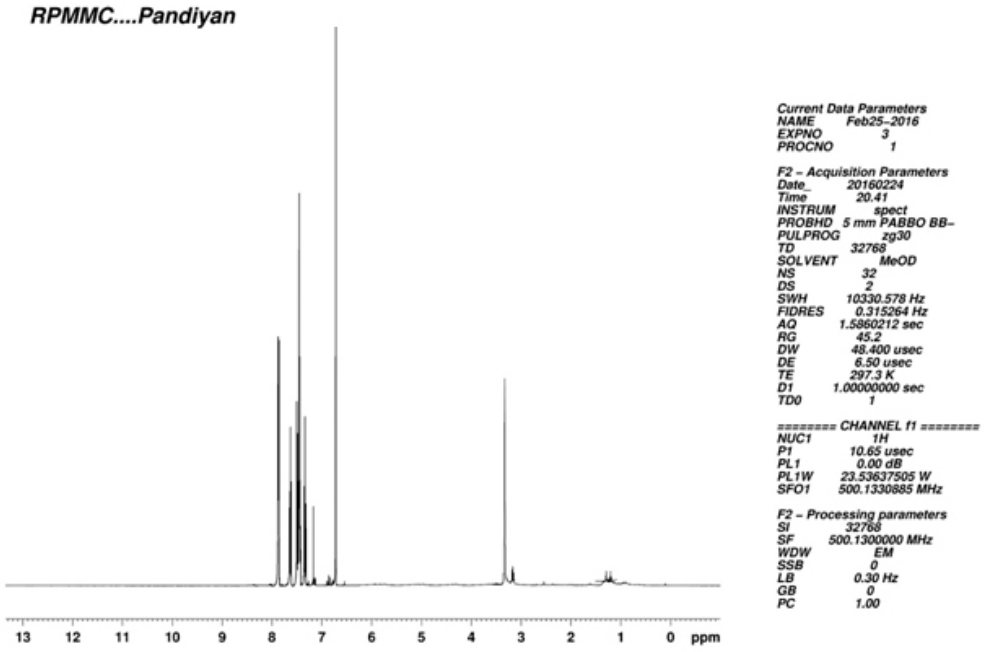
Fig 20. GC MS Spectrum of Isolated compound



The Fig20.Shows the gas chromatogram of isolated compound. It is clearly seen that high RT value of 17.12 and the corresponding molecular weight of 222 m/z which is the flavonoid belongs to the class flavone with the molecular formula $C_{15}H_{10}O_2$

NMR Spectrum

Fig21.^{1}H NMR Spectrum of Isolated compound



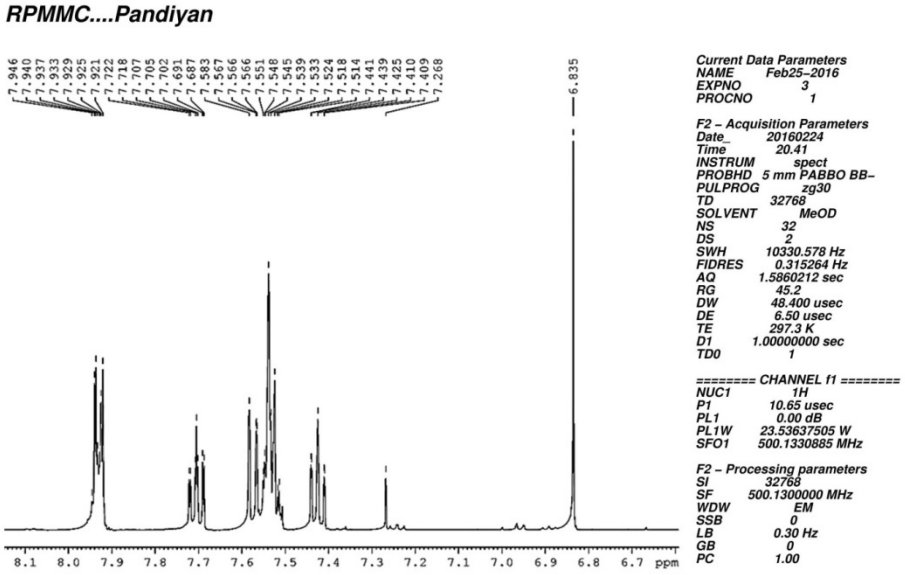
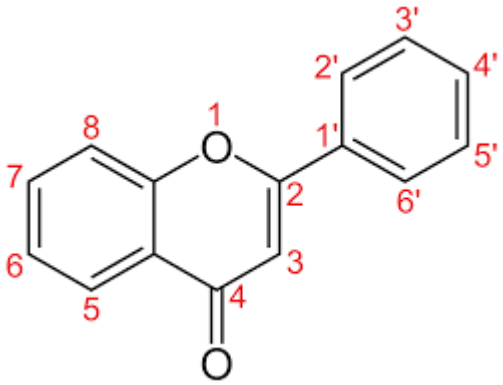


Table 16.^{1}H NMR spectrum of isolated compound:

Type of Proton	δ Value	Peak	No of Protons
Chromene CH	6.8	Singlet	1
a) Aromatic - CH	7.514 - 7.583	Multiplet	5
b) Aromatic - CH	7.921 – 7.946	Doublet	2
ChromeneAr – CH	7.409 – 7.441 & 7.687 -7.722	Triplets	2
Methanol	δ 3.5	Singlet	1

Number of protons = 10
Molecular formula: $C_{15}H_{10}O_2$

Fig 22. Structure of flavone



18. RESULTS OF DOCKING OF AN ISOLATED FLAVONE

Fig23 . Docking pose

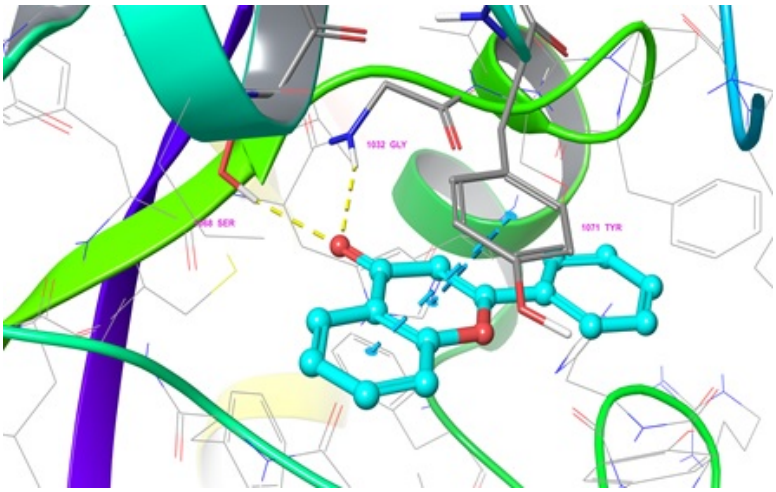
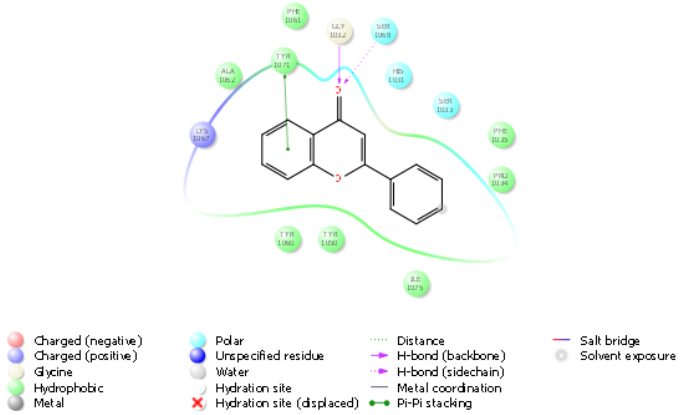


Fig24 . Ligand receptor intraction



Discussion:

The amino acid residue 1032 Glycine and 1068 Serine forms hydrogen bonding with oxygen atom in carbonyl group of chromene ring. Pi bond interaction was formed by tyrosine 1071 in chromene ring which is shown in Fig 23. The hydrogen bonding and the Pi bond interactions lead to a good docking score of -9.538.Tankyrases are ADP-ribosyltransferases that play key roles in various cellular pathways, including the regulation of cell proliferation, and thus, they are promising drug targets for the treatment of cancer. Flavones have been shown to inhibit tankyrases and we report here the discovery of more potent and selective flavone derivatives.[66]

19. *IN VITRO* ANTI-CANCER ACTIVITY OF FLAVONE ON MCF -7 CELL LINE

Table 17.*In vitro* anti-cancer activity of flavone on MCF -7 cell line

Compounds	PERCENTAGE GROWTH					GROWTH INHIBITON IN mg		
	1000µg	100µg	10µg	1µg	0.1µg	GI50	TGI	LC50
Tamoxifen	-29	0	49	89	80	9.2	100.0	100.0
Flavone	-7	40	68	92	92	43.48	100.0	100.0
Average						26.4	100.0	100.0

Fig 25.Percentage growth of MCF-7 Cell

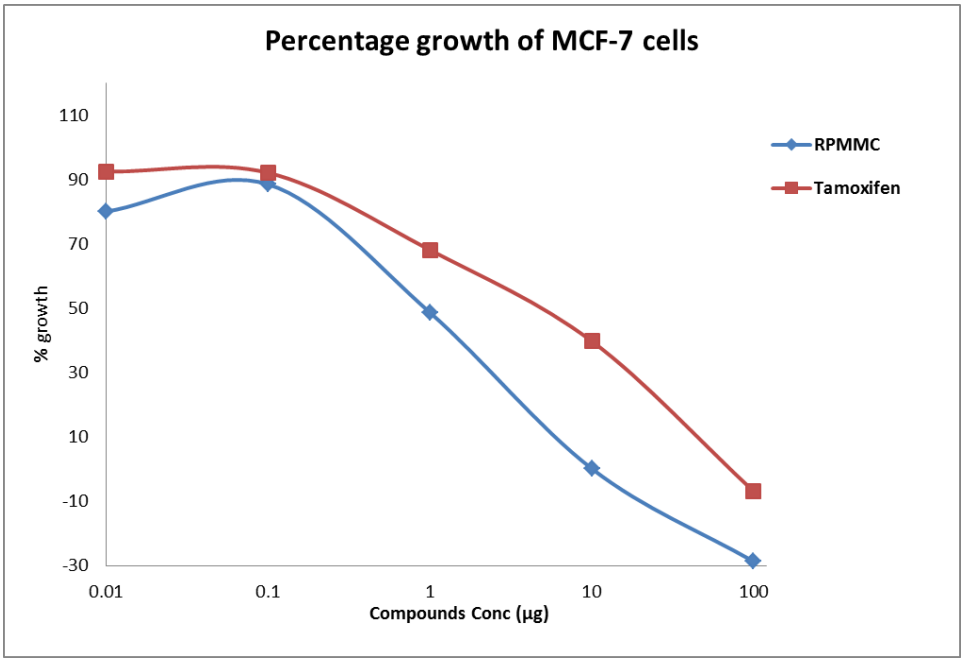


Fig 26.Comparision of $GI_{50}$with the standard tamoxifen

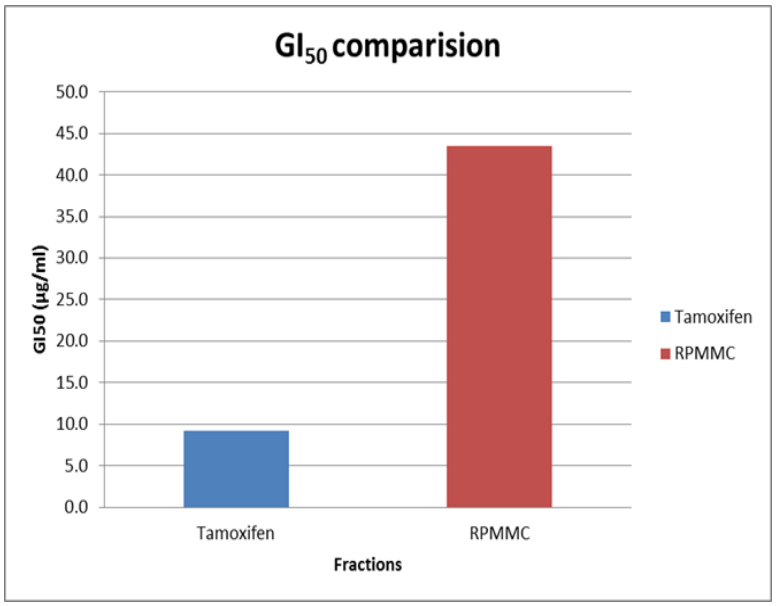
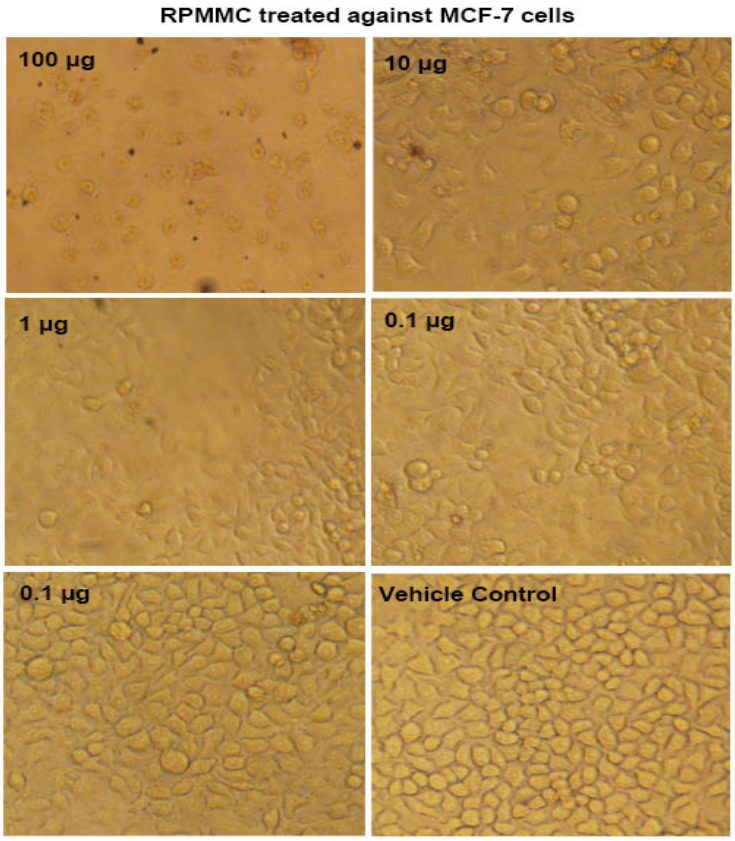


Fig 27.Isolated compound treated against MCF-7 Cell line



DISCUSSION

Table 11 shows that at 100µg of isolated compound flavone, the percentage growth of MCF-7 cells was found to be 40. Flavone exhibits good anticancer activity compared to standard Tamoxifen, which shows dramatic decrease in percentage growth with 0 at 100µg. As discussed earlier in the introduction, the chemical components of medicinal plants mainly possess antioxidant properties that contribute to their anticancer potential. Flavones, isoflavones, flavonoids, anthocyanins, coumarins, lignans, catechins, andisocatechins are the major classes of bioactive constituents responsible for the antioxidant action.[15]Flavonoids may act at the different development stages of malignant tumors byprotecting DNA against oxidative damage, inactivating carcinogens, inhibiting the expressionof the mutagenic genes and enzymes responsible for activating procarcinogenicsubstances,and activating the systems responsible for xenobiotic detoxification.[17]Hence the isolated compound flavone shows good anticancer activity on MCF-7 cell lines.

20. GENE EXPRESSION RESULTS

Table 18. Calculation procedure for MNE

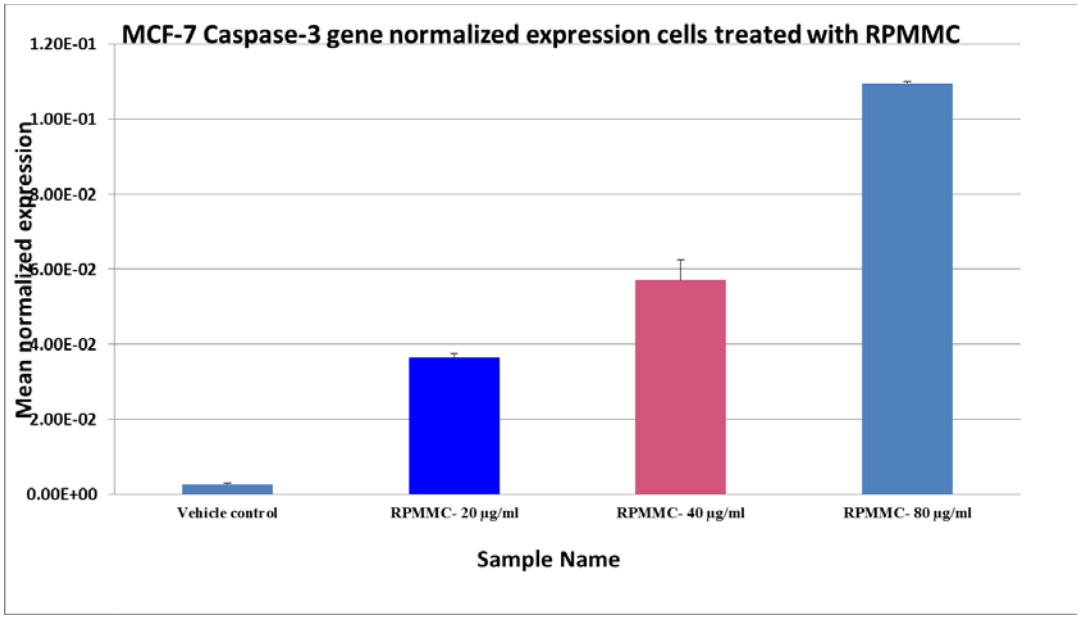
	2
Threshold for SEM in %	20.0

Well	Description	CT of Target Gene	CT of Reference Gene Actin	Normalized Expression
	Vehicle control	33.39	26.73	2.51E-03
		33.39	26.93	2.86E-03
	RPMMC- 20 µg/ml	27.32	24.39	3.76E-02
		27.32	24.29	3.53E-02
	RPMMC- 40 µg/ml	28.47	26.15	5.23E-02
		28.49	26.44	6.24E-02
	RPMMC- 80 µg/ml	27.32	26.06	1.10E-01
		27.32	26.05	1.09E-01

Table 19.Mean of duplicates

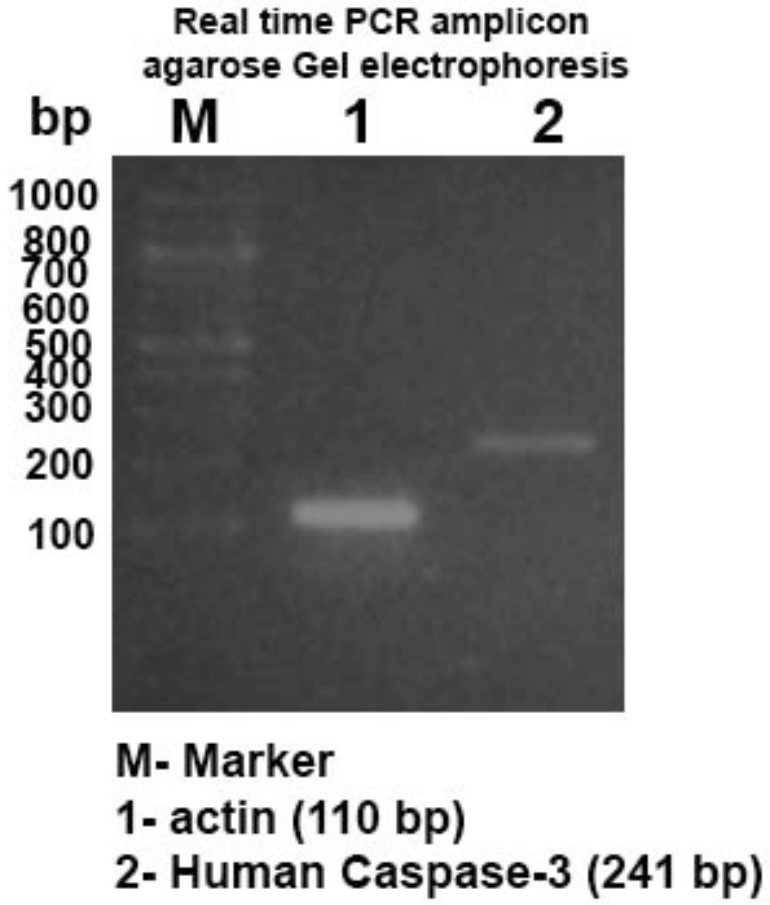
Mean of duplicates			
Description	Mean Normalized Expression	SE of Mean Normalized Expression	SE of Mean Normalized Expression in %
Vehicle control	2.68E-03	1.72E-04	6.42
RPMMC- 20 µg/ml	3.64E-02	1.13E-03	3.10
RPMMC- 40 µg/ml	5.71E-02	5.43E-03	9.51
RPMMC- 80 µg/ml	1.10E-01	3.90E-04	0.36

Fig28. MCFCapase3 gene normalized expression cells treated with isolated compound



J

Fig 29. Real time PCR amplicon agarose Gel electrophoresis

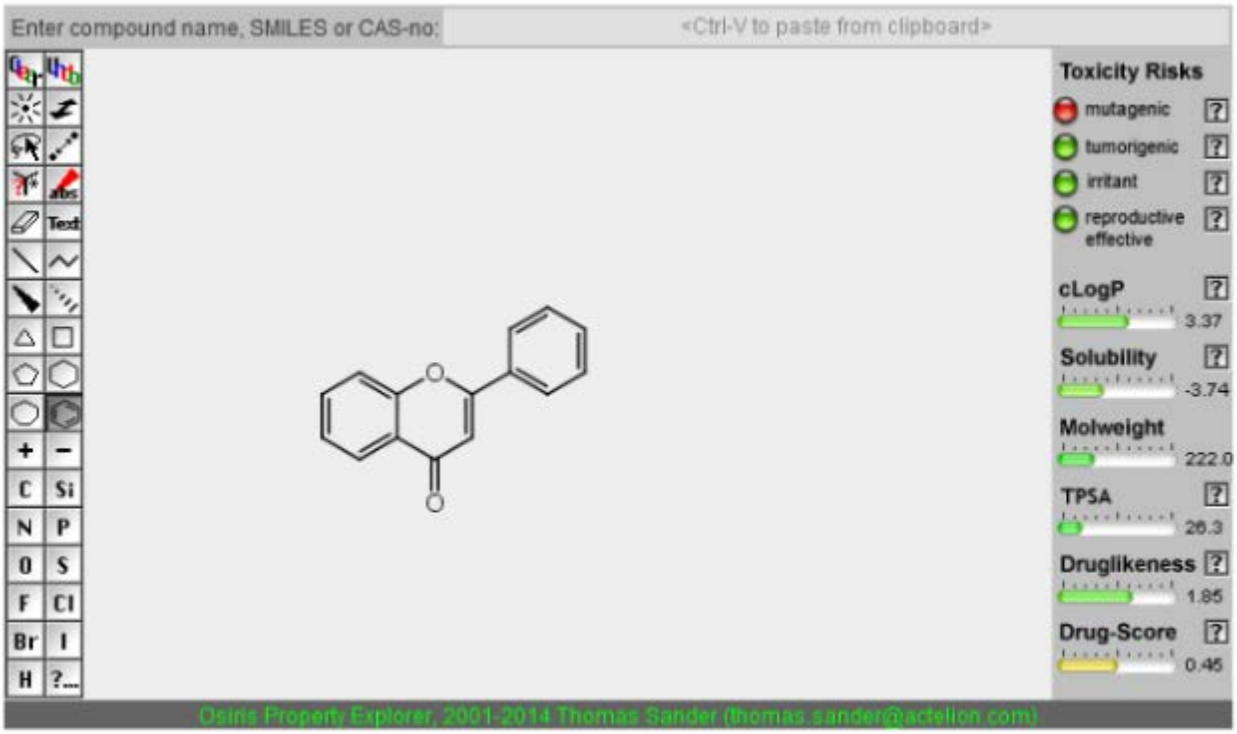


DISCUSSION

The results of gene expression shows that at 80µg/ml there was an increase in the activity of caspase – 3 activity when compared to control gene. The isolated compound, flavone induces apoptosis in MCF-7 cells through caspase -3 activity in a dose dependent manner as show in **fig28.**The previous study supports for our present work that the presence of flavonoids can suppress the cancer cells. Several natural compounds with anticancer effects can induce apoptosis of tumor cells.[67]

21. TOXICITY STUDY OF AN ISOLATED COMPOUND

Fig30 . Isolated compound showing Toxicity Risks



DISCUSSION

The toxicity risk such as mutagenic, tumorigenic ,irritant ,reproductive effective , clog p, solubility, molecular weight , TPSA, drug likeness and drug-score for the specific structure of the isolated compound flavone was determinedfrom the above fig30.It was found that the compound is mutagenic in nature. The other parameters are found to be safe in nature .

22. SUMMARY AND CONCLUSION

The chloroform and ethanol extracts was prepared from the dried powdered roots of *Jatropha gossypiifolia*by hot continuous percolation method using soxhletapparatus.The extracts was subjected to preliminary phytochemical screening and was found that, secondary metabolites such as steroids, alkaloids, glycosides, flavonoids and phenolic compounds. It was found that ethanol extract shows the presence of flavonoids and phenolic compounds and it is absence in chloroform extract.

Bio guided Isolation

***In vitro* anticancer activity**

The chloroform and ethanol extract was subjected to *in vitro* anticancer activity on MCF – 7 cell line. The ethanol extract shows better anti-cancer activity than chloroform extract with the percentage growth of 28.46 on MCF –7cells. Since the present scheme of work is bioguided isolation, the active extract (ethanol extract) was selected for isolation by column chromatography.

Isolation by column chromatography

Ethanol extract was subjected to column chromatography isolation by gradient elution technique. The yellowish green fraction was started eluting fromfractions(chloroform 100%), chloroform: ethylacetate (160:40), (150:50), (100:100) and ethyl acetate 100%. The brown colour fraction was started eluting from fractions (ethylacetate: ethanol) and ethanol 100%.TLC was determined for the above fractions. The fractions with the same $R_f$value were mixed to contain totally of 4 fractions. Fraction 1 (chloroform fraction), fraction 2 (chloroform: ethylacetate), fraction 3 (ethyl acetate: ethanol)and fraction 4 (ethanol 100%).

***In Vitro* anticancer activity of fractions**

All the 4 fractions were subjected to anticancer activity on MCF – 7 cell lines. The fraction 4 (Ethanol 100%) shows the good anticancer activity compared to other fractions against MCF -7 cell line. The percentage growth of MCF-7 cells in fraction (1 -4) was found to be 24.527, 49.132, 36.909, 14.650 respectively. The EC_{50} value was found to be for fractions (1- 4) was found to be 0.00082, 0.1728, 0.00028, 0.00087mg/ml respectively.

This clearly indicates that fraction (4) shows good anticancer activity against MCF-7 cell line when compared to other 3 fractions.

Isolation and Purification of fraction byPreparative HPLC

Ethanol 100% (Fraction 4) is subjected for isolation and purification by Preparative HPLC. In this study, compound isolation is performed effectively by preparative HPLC. Initially UV Spectrum was carried out to find out the wavelength of maximum absorbance (λ_{max}**).** The λ_{max}wasfound to be 237nm as shown in Fig 16.The Analytical HPLC was carried out at the same 237nm. The analysis was carried out under isocratic conditions using a flow rate of 1.0 mL/min at room temperature (25ºC). In Fig:17. The Analytical HPLC shows that Peak 1 has retention time of 2.645 with height 23359 and area of 89.703. The peak 1 was selected for isolation by Preparative HPLC. The peak 1 was selected for isolation by preparative HPLC using Methanol: Water = 90:10 containing 0.1% TFA as mobile phase at a flow rate of 5.4mL /min. Hence the single purified compound was separated effectively. The isolated compound was subjected to qualitative analysis for the test of flavonoid and phenolic compounds. The compound gives the positive test for flavonoid.

Characterization of isolated compound

FT- IR spectra of isolated compound shows that, 2977 cm^{-1} (Aromatic CH stretching), 1730cm^{-1},C=O (Carbonyl group of Chromene ring), 1637cm^{-1}(Aromatic C=C). GC MS Spectrum of isolated compoundFig 20. Shows that high RT value of 17.12 and the corresponding molecular weight of 222 m/z which is a flavone with the molecular formula $C_{15}H_{10}O_2$.^{1}H NMR spectrum of flavone was found to have Aromatic CH at δ7.514 - 7.583 and δ7.921 – δ7.946, multiplets. Chromene CH, δ6.8,singlet. ChromeneAr – CH, 7.409 – 7.441. & 7.687 -7.722,triplets. Hence the isolated compound was found to be flavone.

Docking Study of isolated compound

The crystal structure of human tankyrase 2 in complex with flavone has shown good interaction with the receptor protein with the docking score of -9.538. The interaction with receptor was seen at the carbonyl carbon of chromene ring and the unsaturated part of chromene ring.

***In vitro* anticancer activity of flavone.**

In vitro anticancer activity of flavone was determined by MTT assay.At 100µg of isolated compound flavone, the percentage growth of MCF-7 cells was found to be 40. Hence flavone exhibits good anticancer activity compared to standard Tamoxifen, which shows dramatic decrease in percentage growth with 0 at 100µg.

Gene Expression of flavone by RT PCR

The results of gene expression show that at 80µg/ml there was an increase in the activity of caspase – 3 activity when compared to control gene. The isolated compound, flavone induces apoptosis in MCF-7 cells through caspase -3 activity in a dose dependent manner as show in Fig28**.**The previous study supports for our present work that the presence of flavonoids can suppress the cancer cells.

Toxicity study of isolated compound

The toxicity risk such as mutagenic, tumorigenic,irritant, reproductiveeffective, clog p, solubility, molecular weight , TPSA, drug likeness and drug-score for the specific structure of the isolated compound flavone was determined by using a software OSIRIS. It was found that the compound is mutagenic in nature. The other parameters were found to be safe.

Conclusion

The conclusion of the study present study is the ethanol extract and ethanol fraction of *Jatropha gossypifolia* (root) from column chromatography is proved to have antineoplastic activity. The flavone which is isolated from ethanol fraction is proved to be an effective antineoplastic agent .against MCF-7 cell line.

23.Future Scope

- *Jatropha gossypifolia* is proved to be potential anticancer drug. More research should be carried out to evaluate the *in vivo* anticancer activity on the extracts and isolated compound from this plant.

- Apart from anti-cancer activity there is other pharmacological activity for this plant like neuropharmacological activity, hepatoprotective activity. Research should be carried out to find out the active principles involved in these activities.

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